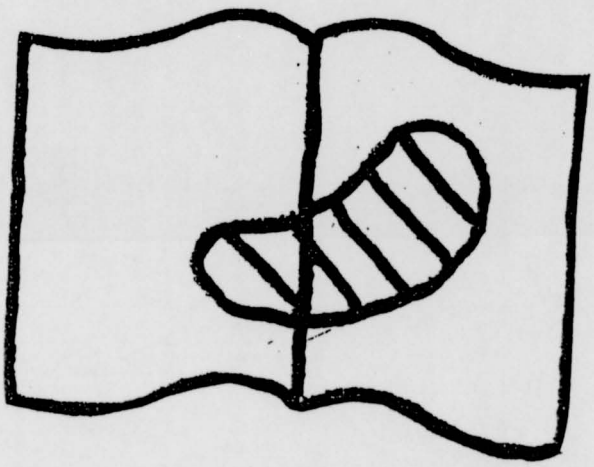

Regulation of methane monooxygenase genes in methanotrophs

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A thesis submitted for the degree of Doctor of Philosophy

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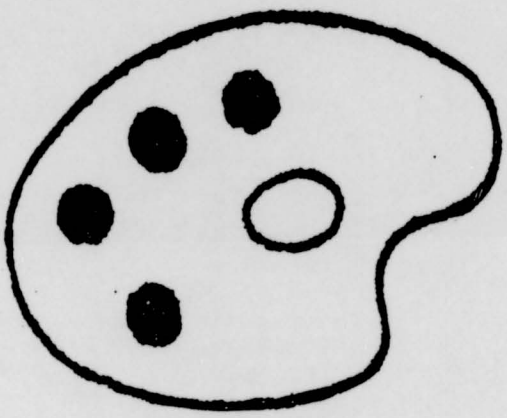

To my wife and family

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trichosporium OB3b.

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I also acknowledge Bettina Gilbert and Allan 'curry king' Nielsen with whom the primer extension experiments were performed.

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I must also thank my family for their help and support over the past few years. I am however, unable to thank my wife Prachi enough for enduring the roller coaster of the past few years, but maybe diamonds will be a start!

Finally, I must acknowledge the financial support of the BBSRC, SGM, and EU-BOMMBE project.

DECLARATION

I declare that all of the work reported in this thesis, is the result of original research conducted by myself (under the supervision of Professor J. C. Murrell). Additional help and information, when obtained, has been referenced.

The primer extension results presented in chapter 3 was published as part of a manuscript entitled: Molecular analysis of the *pmo* (Particulate methane monooxygenase) operons from the two type II methanotrophs. *Applied and Environmental Microbiology* **66.Gilbert, B., McDonald, I. R., Finch, R., Stafford, G. P., Nielsen, A. K. & Murrell, J. C. (2000).**

No work contained within this thesis has been previously submitted for any other degree.

Graham P. Stafford

Abbreviations

	Description
A	Absorbance
Ap	Ampicillin
AMO	Ammonia monooxygenase
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
CBC	Copper Binding Compound
Cm	Chloramphenicol
CTAB	Cetyltrimethyl ammonium bromide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTT	D, L-Dithiothreitol
EBP	Enhancer binding protein
EDTA	Ethylenediaminetetra-acetic acid
EPR	Electroparamagnetic resonance
g	gram
GFP	Green fluorescent protein
h	hour
IEF	Isoelectric Focus
IHF	Integration host factor
IS	Insertion sequence
kbp	kilo base pairs
kDa	kilo Daltons
K_m	Michaelis constant
l	litre
LB	Luria broth
M	Molar
MDH	Methanol Dehydrogenase
Mg	milligram
Min	minute
Ml	millilitre

MMO	Methane monooxygenase
mol	mole
mM	millimole
mRNA	messenger RNA
NA	Nutrient agar
NAD/NADH	Nicotinamide adenine dinucleotide (oxidised/reduced)
nmol	nanomole
NMS	Nitrate mineral salts
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide gel eletrophoresis
PCR	Polymerase chain reaction
pMMO	particulate methane monooxygenase
RNA	Ribonucleic acid
RPM	Revolutions per minute
RRNA	ribosomal RNA
RuMP	Ribulose monophosphate
SDS	Sodium dodecyl sulfate
sMMO	soluble methane monooxygenase
TAE	Tris-Acetate EDTA
TBE	Tris-Borate EDTA
TBST	Tris buffered salt, plus Tween 20
Tc	Tetracycline
TCE	Trichloroethylene
TEMED	*N, N, N, N'*-tetramethylethylenediamine
Tg	Terragrams
TRNA	transfer RNA
UV	Ultra violet
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume

Summary

Methanotrophs are bacteria capable of using methane as their sole carbon and energy source. Oxidation of methane by *Methylosinus trichosporium* OB3b is catalysed by the particulate (pMMO) and soluble (sMMO) methane monooxygenase enzymes. Transcription of the genes encoding pMMO (*pmo*) occurs at high copper-to-biomass ratio and sMMO (*mmo*) at low copper-to-biomass ratios. The aim of this work was to study the regulation of methane monooxygenase gene expression in response to copper ions- the "copper switch".

Primer extension and RT-PCR revealed putative transcriptional start sites 5' of the *pmoC* genes from *Methylocystis* sp. strain M and *Methylosinus trichosporium* OB3b that corresponded to σ^{70}-promoters. The *pmo* operons from both organisms possessed tandem promoters 5' of *pmoC* suggesting differential expression of the two sets of *pmo* genes. A qualitative analysis of *pmo* transcription from *M. trichosporium* OB3b revealed that the *pmo* operon is transcribed at both high and low copper ion concentrations when *M. trichosporium* OB3b was grown on methane or methanol.

The gene encoding σ^{N} (*rpoN*) was cloned and sequenced, since previously Nielsen *et al.*, (*Mol Microbiol* (1997) *25*, 399-409) had shown that transcription of the *mmo* genes from *M. trichosporium* OB3b was shown to proceed from a σ^{N}-type promoter. Marker-exchange mutagenesis of *rpoN* verified its role in transcription of the *mmo* operon and also suggested its requirement for transcription of both nitrogen fixation and nitrate reduction genes. Sequencing of the region 5' of the *mmo* cluster (Ian McDonald, unpublished) revealed a gene possessing homology with σ^{N}-dependent transcriptional activators, named *mmoR*. Mutation of *mmoR* by marker-exchange mutagenesis showed it was also required for expression of the *mmo* operon. These data have allowed an improved model for the regulation of the *pmo* and *mmo* gene clusters by copper to be proposed.

Chapter 1

Introduction

1.1 Introduction to methanotrophs

Methanotrophs are a unique group of aerobic bacteria that are capable of utilising methane as their sole carbon and energy source (Hanson and Hanson, 1996). Some methanotrophs are also capable of using methanol as a carbon and energy source, but are considered obligate in their requirement for one-carbon compounds and cannot be grown on multi-carbon substrates.

1.1.1 Taxonomy

The basis for the current classification of methanotrophs was established after the pioneering work of Whittenbury *et al.*, (1970) in which over 100 methane-utilising bacteria were isolated and classified. These authors proposed the grouping of methanotrophs into five genera: *Methylomonas, Methylobacter, Methylococcus, Methylocystis* and *Methylosinus*. A sixth genus: *Methylomicrobium* was added after the work of Bowman *et al.*, (1993,1995). Recent years have seen the description of several new genera: the acidophiles *Methylocella* (Dedysh *et al.*, 2000) and *Methylocapsa* (Dedysh *et al.*, 2002); *Methylosarcina* (Wise *et al.*, 2001); the psychrophile *Methylosphaera* (Bowman *et al.*, 1997) and the thermophile *Methylocaldum* (Bodrossy *et al.*, 1997). The methanotrophs have been grouped into two types based on their biochemistry, morphology and phylogeny (Hanson and Hanson, 1996). The Type I methanotrophs include the γ-*Proteobacterial* genera *Methylococcus, Methylocaldum, Methylomicrobium, Methylomonas, Methylosarcina, Methylosphaera*; and the Type II (α-*Proteobacterial*) methanotrophs: *Methylosinus, Methylocystis, Methylocapsa* and *Methylocella.* The major distinguishing characteristics of the two types are shown in table 1.1.

Table 1.1 Characteristics of Type I and Type II methanotrophs. Adapted from Hanson and Hanson, (1996).

Characteristic	Type I	Type II
G +C content of DNA (mol%)	49-60	62-67
Membrane arrangement		
Bundles/ vesicular disks	+	-
Peripheral membranes	-	+
Resting cells	Cyst	Cyst or exospore
Complete TCA-cycle	-	+
Predominant fatty acid carbon chain length	16	18
RuMP pathway	+	-
Serine pathway	-	+
Proteobacterial subdivision	γ	α

1.1.2 Ecological significance

Methane is the most abundant organic gas in the atmosphere with the total sources of methane estimated at 520 Tg per year (Hanson and Hanson, 1996). The concentration of methane in the atmosphere has increased in the last 300 years with the most likely cause being human activity, but more importantly it is 26 times more effective as a greenhouse gas than carbon dioxide (Hogan, 1991; Lelieveld *et al.*, 1993). These organisms have thus been of great interest, as it is important to gain an understanding of the effects of global warming on the net sinks and sources of methane. Indeed, methanotrophs play a major role in the global methane cycle as they oxidise the methane produced by methanogens in anaerobic freshwater and wetland sediments. They have also been isolated from an increasingly diverse set of environments, including hot springs (Bodrossy *et al.*, 1995;Whittenbury *et al*, 1970); soda lakes (Khmelenina *et al.*, 1997); acidic peat bogs (Dedysh *et al.*, 2000), and the Antarctic tundra (Bowman *et al.*, 1997) indicating their widespread distribution and potential significance in many environments.

A second group of aerobic methanotrophs, which has not as yet been identified, exhibit high-affinity methane oxidation kinetics, are capable of oxidising atmospheric levels of methane (1-2 ppm) and may play a role in reducing the effects of this gas in the atmosphere (Bender & Conrad, 1992; Holmes *et al.*, 1999). It is also known that anaerobic methane oxidation is an important process in marine environments, although little is known about the biochemistry or physiology of the

micro-organisms performing this oxidation and their identity is only now being partly resolved (Orphan *et al.,* 2001).

1.1.3 Commercial applications

The last 30 years has seen a considerable amount of interest in the methanotrophs due to their potential for commercial applications. At the simplest level, a large amount of biomass could be generated from methanotrophic cultures using methane as their sole carbon and energy source. Indeed, *Methylococcus capsulatus* Bath is used in the production of single-cell-protein products for the salmon industry in Norway, using natural gas as the substrate for their growth (Larsen and Joergensen, 1996). *Methylococcus capsulatus* Bath constitutes approximately 95 % of bacteria in the bioreactors used for production of this feedstock, with the remaining 5 % consisting of heterotrophs specialised in the removal of toxic products resulting from the co-oxidation of trace amounts of other carbon compounds in natural gas by pMMO and sMMO (Fjellibirkeland *et al.,* 2001). In this way, large amounts of protein can be produced relatively cheaply, making this bacterium an attractive organism for the heterologous expression of proteins of commercial interest. Towards this end, recent research has also focussed on protein secretion mechanisms in *Methylococus capsulatus* Bath and has resulted in the isolation of several outer membrane proteins, which may allow expression of foreign proteins in the future. Of particular interest is the 60 kDa MopE protein of *M. capsulatus* Bath, which is cleaved at a specific site and released into the cell culture medium in high concentrations (20 mg/l) (Fjellibirkeland *et al.,* 2001).

However, much research has focussed on the ability of methanotrophs to co-metabolise several low-molecular weight chlorinated hydrocarbons, notably trichloroethylene (TCE). These compounds are used as solvents in many processes such as degreasing metals and electronic components, semiconductor manufacturing, dry cleaning and as fumigants for the control of pests. Their widespread use and careless disposal has made them one of the most abundant groundwater pollutants in the United States (Ensley, 1991). A pure culture of the methanotroph *Methylosinus trichosporium* OB3b has been shown to be at least two orders of magnitude more efficient at degrading TCE than other bacteria, provided that it was expressing the soluble form of its methane monooxygenase enzyme (Tsien *et al.,* 1989). Several

bioreactor-based processes utilising mixed cultures of methanotrophs and heterotrophs for the specific removal of TCE have been described (reviewed in Hanson and Hanson, 1996; Sulllivan *et al.,* 1998). However, several problems have been encountered: 1) Competition between solvents and methane for the active site of methane monooxygenase; 2) Inactivation of sMMO activity by high levels of copper; 3) A gradual decrease in the efficiency of reactors due to toxic effects of the solvents being treated. The possibility of *in situ* bioremediation has also been investigated and the supply of a methane and air mixture to a river site stimulated an expansion in the population of TCE-degrading methanotroph species (Bowman *et al.,* 1993).

The recent discovery of several thermophilic strains of methanotroph by Bodrossy *et al.,* (1995) with TCE degrading abilities, which are more resistant to copper, may also increase the possibility of the use of these organisms in the production of chemicals based on biotransformations carried out by methanotrophs. The conversion of methane to methanol by methanotrophs and its subsequent extraction has long been of interest, and these thermophilic bacteria may lead to the development of a commercially viable process for the biological production of methanol in the future.

1.2 Methane oxidation pathway

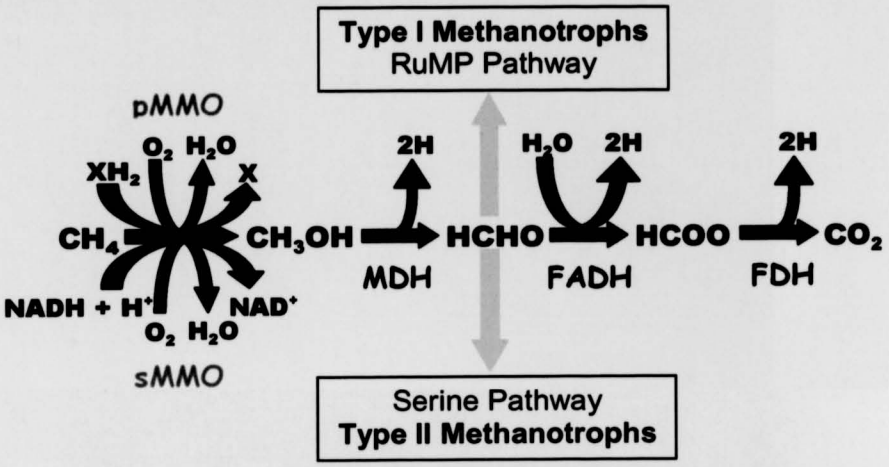


Figure 1.1 Methane oxidation pathway. The two forms of the methane monooxygenase, pMMO and sMMO use different electron donors. NADH is the source of reducing power for sMMO, but the natural electron donor for pMMO is unknown. Methane is converted to methanol by methanol dehydrogenase (MDH). 2H represents generation of reducing equivalents. Approximately 50% of carbon is assimilated at the level of formaldehyde via the Ribulose monophosphate pathway (Type I) or the Serine pathway (Type II methanotrophs). The remaining 50% is oxidised to formate by Formaldehyde dehydrogenase (FADH) followed by oxidation by formate dehydrogenase (FDH) releasing CO_2 to the atmosphere.

The pathway for the oxidation of methane is shown in figure 1.1. Methane is oxidised by methanotrophs to CO_2 via methanol, formaldehyde and formate. Approximately 50 % of this carbon is assimilated into cell biomass at the level of formaldehyde and the remaining 50 % lost to the atmosphere as CO_2. The oxidation of formaldehyde to CO_2 generates reducing power for biosynthesis and the initial methane oxidation step. Recently, an alternative route for the dissimilation of formaldehyde has been found in methanotrophs (Chistoserdova *et al.*,1998; Vorholt, *et al.*,1999) revealing that there are several routes for the removal of the toxic intermediate formaldehyde from the cell (Murrell *et al.*, 2001).

The first step in the oxidation of methane is its conversion to methanol. This reaction is catalysed by one of two distinct methane monooxygenase enzymes: a particulate, membrane-bound methane-monooxygenase (pMMO) or a soluble, cytoplasmic enzyme (sMMO). The pMMO has been found in all methanotrophs isolated thus far, with the possible exception of *Methylocella palustris* (Dedysh *et al.*, 2000), and the sMMO in several, but not all genera of methanotrophs. The pMMO is expressed under growth conditions where the copper-to-biomass ratio is high.

Removing copper from the growth medium of methanotrophs possessing only the pMMO enzyme such as *Methylomicrobium album* BG8 results in poor growth (Berson and Lidstrom, 1997).

1.3 Particulate methane monooxygenase (pMMO)

1.3.1 Biochemistry of the particulate methane monooxygenase (pMMO)

The pMMO enzyme has a relatively narrow substrate range, oxidizing alkanes and alkenes up to five carbons in length but it is not able to oxidise aromatic compounds such as naphthalene (Burrows *et al.,* 1984). This is important since naphthalene can be oxidised by the sMMO enzyme and thus an assay has been developed based upon the detection of the oxidation products of naphthalene (1 and 2-naphthol) which allows the form of MMO being expressed to be determined (Brusseau *et al.,*1991: Section 2.23). However, the pMMO of *Methylosinus trichosporium* OB3b is capable of efficient degradation of TCE at elevated copper levels (20 μM), indicating that it may be of use for bioremediation of this compound (Lontoh and Semrau, 1998).

The pMMO from *Methylococcus capsulatus* Bath was the first to be partially purified by solubulizing the enzyme from membranes using β-D dodecyl maltoside (Smith and Dalton, 1989) and it is the pMMO from this organism which has been the chief focus of subsequent research. The purified pMMO enzyme from *Methylococus capsulatus* Bath consists of three subunits of approximately 47, 27 and 23 kDa arranged in a 1:1:1 ratio (Zahn and Dispirito, 1996; Nguyen *et al.,* 1998). The active site of this enzyme is believed to reside within the 47 and 27 kDa subunits, as shown by ^{14}C-labelling experiments using the suicide substrate acetylene (Zahn and Dispirito, 1996; Prior and Dalton, 1985). The active form of pMMO has been reported to contain 14.5 copper ions (Zahn and Dispirito, 1996) and between 12-15 copper ions by Nguyen *et al.,* (1998). It probably also contains 2.5 iron atoms per active enzyme complex (Zahn and Dispirito, 1996) although this is disputed by Nguyen *et al.,* (1998). The pMMO from *Methylosinus trichosporium* OB3b has recently been purified and shows similar properties (Takeguchi *et al.,* 1999a,b). The presence of iron in pMMO from *Methylococcus capsulatus* Bath is supported by the findings of Takeguchi *et al.,* (1999a,b) who found that the active pMMO from *Methylosinus trichosporium* OB3b contained 0.9 iron atoms and 12.8 copper atoms.

Interestingly, as long ago as 1983, evidence was available to suggest the importance of iron to the pMMO enzyme: Stanley *et al.,* (1983) observed that pMMO activity was severely inhibited by both ferric and ferrous iron chelators. The exact nature of the copper and iron in the active site of pMMO is unclear. Nguyen *et al.,* (1996) proposed that copper existed as a tri-nuclear copper cluster in the active site of pMMO. However, EPR studies by Zahn and Dispirito (1996) suggested the presence of a type 2 copper centre and non-heme iron in an Fe[II]-Fe[II] centre, a single Fe[II] centre or an Fe[II]-copper centre. In addition, Nguyen *et al.,* (1996) and Takeguchi *et al.,* (1999a) have shown that approximately 70% of the copper present in membranes from pMMO expressing cells is silent in electron spin resonance spectra, indicating that 70% of the copper is in the form of copper [I].

Purified pMMO also contained 3-7% cytochrome b-559/569, which caused loss of activity when removed (Zahn and Dispirito, 1996). However, Nguyen *et al.,* (1998) failed to replicate this observation. Thus, it is difficult to assign the physiological supply of reducing power to pMMO. However, duroquinol and $NADH_2$ can be used as artificial reductants (Zahn & Dispirito, 1996). Indeed the treatment of pMMO from *Methylosinus trichosporium* OB3b with duroquinol results in the loss of the iron signal from EPR spectra, indicating that an iron atom is contained within the active site of pMMO in this organism (Takeguchi *et al.,* 1999a,b). A model for the oxidation of methane by pMMO using the artificial reductant duroquinol was proposed by Zahn and Dispirito (1996) and is shown in Figure 1.2.

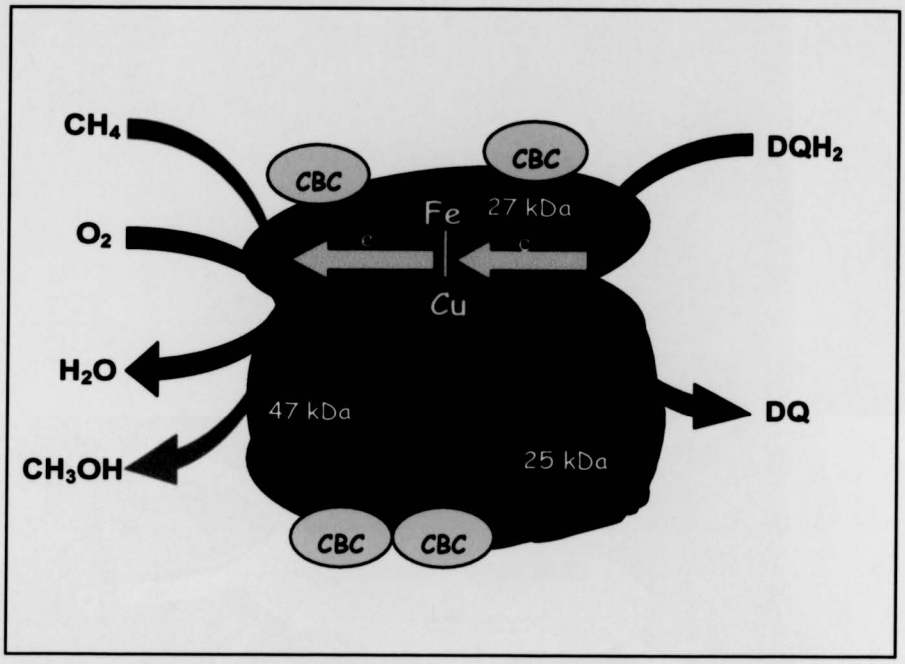


Figure 1.2 Proposed model for the mechanism of methane oxidation by the pMMO using duroquinol (DQH_2) as the reductant. The pMMO enzyme is illustrated in association with the copper binding compounds (CBC) (see section 1.3.1and 1.10.1). Adapted from Zahn and Dispirito, (1996).

The majority of the copper associated with purified pMMO and pMMO containing membranes, 12-15 ions, is believed to be bound to copper-binding compounds (CBCs) (Zahn and Dispirito, 1996). These are small peptide molecules, which have been found in association with pMMO containing membranes, and in the medium of copper starved cells of both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath (Zahn and Dispirito, 1996; Dispirito *et al.,* 1998; Tellez *et al.,* 1998). Two forms of the CBCs have been discovered: $CBC\text{-}_{L1}$ with a molecular mass of 1,218 Da and has been found in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b; $CBC\text{-}_{L2}$ with a molecular mass of 779 Da is found only in *Methylosinus trichosporium* OB3b (Dispirito *et al.,* 1998). The known primary structure of the CBCs consists of amino acids not normally associated with strong copper complexes, and therefore they are thought to be highly modified by the addition of unknown functional groups, which allow the binding of 2-3 copper atoms per molecule (Dispirito *et al.,* 1998). The physiological role of the CBCs is still

unclear, but it possible that they are part of a pMMO-specific copper acquisition system (Discussed in section 1.10.1) or that they are in some way directly involved in methane oxidation by pMMO (Dispirito *et al.*, 1998).

Interestingly, pMMO shares several similarities with the ammonia monooxygenase (AMO) from ammonia-oxidising bacteria, such as *Nitrosomonas europaea*, at the protein and genetic level. Both enzymes are membrane associated, have similar substrate ranges and both oxidise methane and ammonia (although with different K_m values). Both are inhibited by acetylene, which binds to the homologous protein subunits in both enzymes (Zahn and Dispirito, 1996; McTavish *et al.*, 1993). Evidence also suggests that both AMO and pMMO are stabilised by copper and that both contain an iron centre (Nguyen *et al.*, 1994; Zahn and Dispirito, 1996; Ensign *et al.*, 1993). Indeed. Holmes *et al.*, (1995) have proposed that the genes encoding pMMO and AMO are evolutionarily related.

1.3.2 Molecular biology and genetics of pMMO

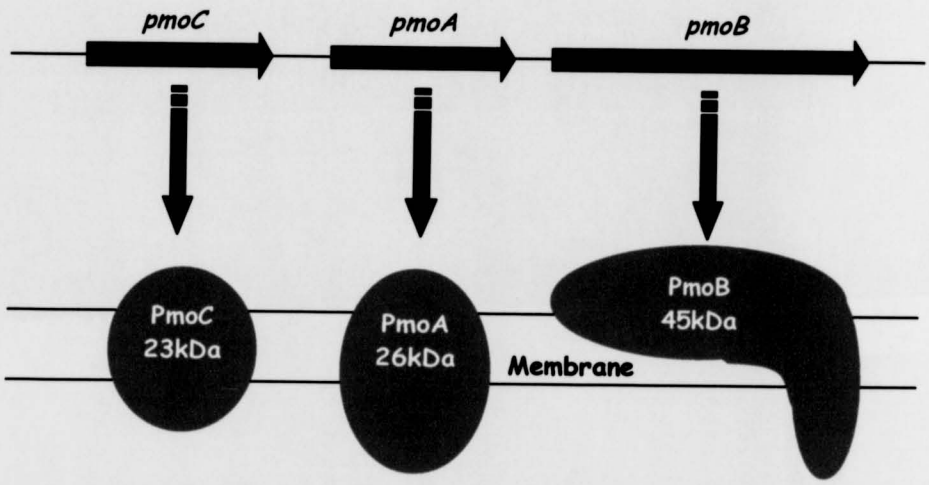


Figure 1.3 Genetic organisation of pMMO operons from methanotrophs.

Growth of methanotrophs in high copper media results in the expression of the pMMO enzyme (Stanley *et al.*, 1983). Not until N-terminal sequencing of the 45 kDa subunit of the pMMO from *Methylococcus capsulatus* Bath was performed was the cloning of the gene encoding this protein, *pmoB*, possible (Semrau *et al.*, 1995). The

gene encoding this protein was named *pmoB* due to the identity of its derived amino acid sequence (42.9 %) with the *amoB* gene from *Nitrosomonas europaea* (Semrau *et al.*, 1995). Subsequently the three genes encoding the pMMO subunits were shown to be present in the genome of *Methylococcus capsulatus* Bath in the order *pmoCAB*, encoding proteins of 23, 27 and 45 kDa respectively (Figure 1.3) (Stolyar *et al.*, 1999; Semrau *et al.*, 1995). The corresponding genes from the ammonia utilisers are known to be present in the same order, *amoCAB*, in two complete copies in the genome of *Nitrosomonas europaea* (Mctavish *et al.*, 1993) and several other ammonia-oxidising bacteria (Sayavedra-Soto *et al.*, 1996). Stolyar *et al.*, (1999) reported the cloning and sequencing of two complete copies of the *pmoCAB* genes of *Methylococcus capsulatus* Bath that contained only 13 nucleotide differences over the 3,183 bp of the *pmoCAB* coding region. This organism also contains a third copy of *pmoC* (Stolyar *et al.*, 1999). The duplication of *pmo* genes is now also known to occur in *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (Gilbert *et al.*, 2000).

One complete copy of the *pmo* genes from *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M have now been sequenced by Gilbert *et al.*, (2000). A comparison of both the nucleotide and derived amino-acid sequences from these organisms shows a high degree of similarity (Table 1.2) which may be expected since they rely on methane as carbon and energy source.

Table 1.2 Comparison of *pmo* genes from *M.trichosporium* OB3b, *Methylocystis* sp. strain M, and *M. capsulatus* Bath.
Copy 1 of *pmo* from *Methylococcus capsulatus* Bath was used for this analysis.

Comparison	*pmoC*		*pmoA*		*pmoB*	
	% DNA identity	% aa identity	% DNA identity	% aa identity	% DNA identity	% aa identity
M.trichosporium OB3b v *Methylocystis* sp. strain M	86	86	84	87	83	81
M.trichosporium OB3b v *M. capsulatus* Bath	75	60	69	59	63	46
Methylocystis sp. strain M v *M. capsulatus* Bath	75	61	70	58	62	46

The role of the two copies of the *pmo* operons is unclear, but two studies by Stolyar *et al.,* (1999, 2001) have answered some questions. Firstly, individual insertion mutants were made in the *pmoCAB* genes of both copies of the *pmo* genes from *Methylococcus capsulatus* Bath. Copy 1 mutants showed approximately two-thirds of the wild-type methane oxidation activities while copy 2 mutants possessed only one-third activity of the wild-type strain (Stolyar *et al.,* 1999). However, neither mutant had significantly lower growth rates under the growth conditions tested. However, these researchers were not able to obtain a double mutant in both *pmo* clusters or in the third copy of *pmoC*. This suggests that the cells require one copy of the *pmoCAB* cluster to be expressed for normal growth.

1.4 The soluble methane monooxygenase (sMMO)

Some methanotrophs contain a second form of methane monooxygenase, the soluble methane monooxygenase (sMMO) that is expressed at low copper-to-biomass ratios (Stanley *et al.,* 1983). Unlike pMMO, sMMO has an extremely wide substrate range and is capable of co-oxidising alkanes, alkenes and several aromatic compounds such as naphthalene, making it an attractive enzyme for biotransformation reactions (Sullivan *et al.,* 1998) since large amounts of the enzyme can be produced using low-cost feedstocks. It is also attractive for bioremediation applications as it is capable of degradation of the groundwater pollutant TCE (introduced in section 1.1.3).

1.4.1 Biochemistry of sMMO

The sMMO enzyme has been purified from several methanotrophs including '*Methylobacterium*' sp. strain CRL-26 (Patel & Savas, 1987), *Methylosinus sporium* 5 (Pilkington & Dalton, 1991) and *Methylocystis* sp. strain M (Nakajima *et al.,* 1992). But the sMMO from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath have been most extensively studied. The sMMO enzyme complex comprises three components: protein A, the hydroxylase; protein B, regulatory protein; protein C, the reductase component (Figure 1.4).

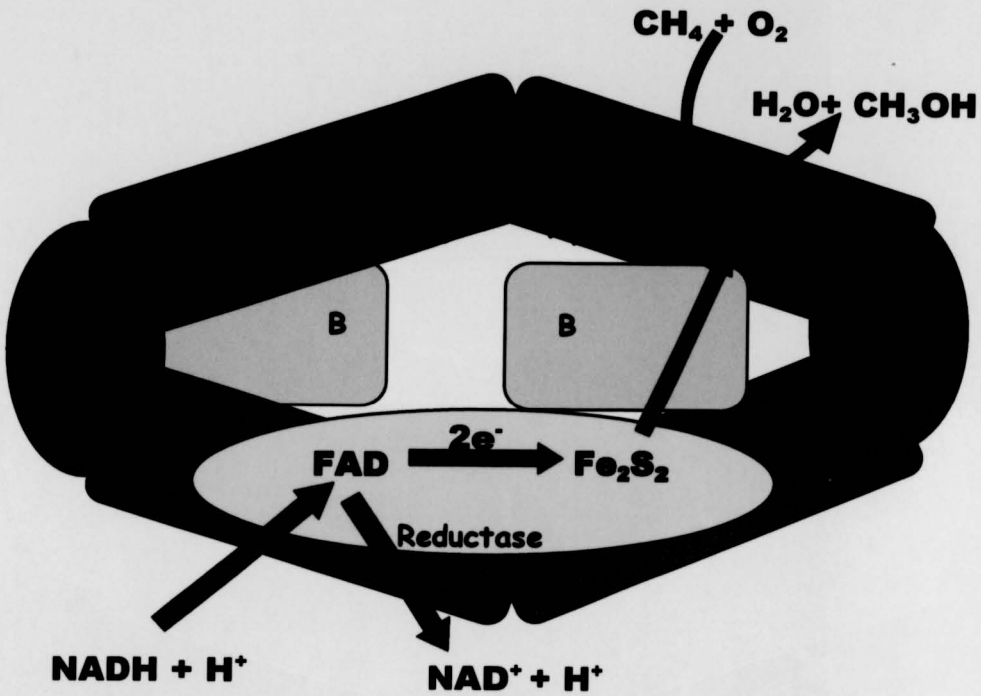


Figure 1.4 Cartoon of the soluble methane monooxygenase complex from *Methylococcus capsulatus* Bath (after Rosenzweig *et al.*, 1994) showing the proposed pathway of electron flow from NADH to the di-iron centre and active site of the α-subunit of sMMO-Hydroxylase (protein A).

Protein A, the hydroxylase of sMMO in *Methylosinus trichosporium* OB3b is composed of 3 subunits of 60, 45 and 20 kDa, arranged as a heterotrimer in an $\alpha_2\beta_2\gamma_2$ configuration. The α-subunit contains a non-heme bis-μ-hydroxo-bridged binuclear iron centre which forms the active site where methane is converted to methanol. The crystal structure of the hydroxylase components of sMMO from *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b has been solved to 1.7 Å and 2 Å respectively (Rosenzweig *et al.*, 1993; Elango *et al.*, 1997). These data revealed that the di-iron centre lies approximately 12 Å below the 'floor' of two canyon regions formed by the α and β subunits. The iron-binding motif of the sMMO hydroxylase: Glu-X-X-His; is conserved among the derived amino acid sequences of all methanotrophs containing an sMMO (McDonald *et al.*, 1997; Shigematsu *et al.*, 1999) and is closely related to the iron-binding motif in the R2 subunit of Ribonucleotide reductase from *Escherichia coli* (Nordlund *et al.*, 1992). The catalytic

cycle for the oxidation of methane to methanol at the di-iron site has been reviewed by Lipscomb (1994).

In common with other multi-component hydroxylase enzymes, sMMO contains a small regulatory protein, protein B (reviewed in Murrell *et al.,* 2001, Green & Dalton, 1985). At low concentrations of protein B, the hydroxylase is converted from an oxidase to a hydroxylase and at saturating concentrations (of protein B) dramatically increases the rates of formation of intermediates and accelerates catalysis of methane to methanol (Lee and Lipscomb, 1999). The sMMO from *Methylococcus capsulatus* Bath is inactive in the absence of protein B whilst *Methylosinus trichosporium* OB3b sMMO functions at a reduced level in the absence of protein B (reviewed in Sullivan *et al.,* 1998). Protein B may be regulated by site-specific proteolysis at its amino terminus (Lloyd *et al.,* 1997). Protein B has also been suggested to play a number of roles including shifting the redox potential of the hydroxylase, varying the rate of product formation when the hydroxylase is functioning via the H_2O_2 shunt, but most importantly causes an increase in the rate of reaction of sMMO by 150-fold (Fox *et al.,* 1991). The structure of protein B has now been elucidated from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath by NMR and is suggested to dock with the hydroxylase protein in the canyon between the α and β subunits of protein A (Walters *et al.,* 1999; Fox *et al.,* 1991).

The third component of sMMO is an NADH-dependent reductase (protein C). It is 39 kDa iron-sulfur flavoprotein containing both FAD and a [2Fe-2S] cluster. Its function is in the transfer of electrons from NADH to the di-iron site of the hydroxylase via its [2Fe-2S] cluster (Lund *et al.,* 1985). It has been shown to bind to the β-subunit of the hydroxylase and may thus play a regulatory role on the hydroxylase via conformational change (Fox *et al.,* 1991). The activity of the reductase is known to be inhibited by copper [II] ions by causing the precipitation of the reductase component (Green *et al.,* 1985; Jahng and Wood, 1996), however this was not observed using Cu [I] indicating that this may not be of significance *in vivo.*

1.4.2 Molecular Biology of sMMO

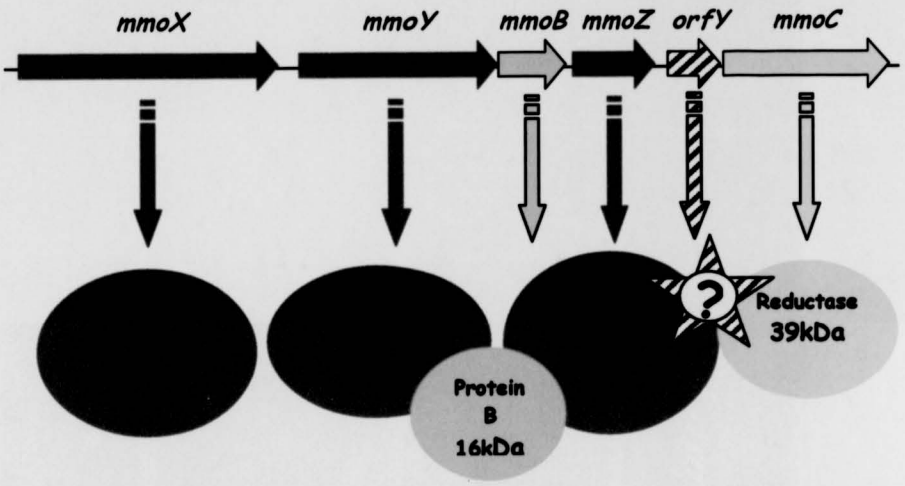


Figure 1.5 Organisation of the genes encoding the sMMO enzyme in methanotrophs.

The genes encoding sMMO have been cloned and sequenced from several methanotrophs including: *Methylococcus capsulatus* Bath (Stainthorpe *et al.*, 1990 a,b), *Methylosinus trichosporium* OB3b (Cardy *et al.*,1991), *Methylocystis* sp. strain M (McDonald *et al.*,1997), *Methylomonas* sp. KSWIII and *Methylomonas* sp. KSPIII (Shigematsu *et al.*,1999). In all cases the genes encoding the sMMO are contained in a six gene operon as illustrated in Figure 1.5. The genes *mmoX, mmoY* and *mmoZ* encode the α, β, and γ subunits of the sMMO hydroxylase. Whilst *mmoB* and *mmoC* encode protein B and the reductase component respectively. A sixth gene, *orfY*, with a coding capacity of 12 kDa is present in all *mmo* clusters sequenced thus far, but has no known function (McDonald *et al.*, 1997; Lloyd, 1997).

A comparison of the *mmo* gene clusters from six methanotrophs (Table 1.3) shows that there is a high degree of conservation among all six genes. However, it is clear that the highest degree of conservation is present for the *mmoX* gene at both the DNA and amino acid sequence level (Shigematsu *et al.*, 1999). This is not surprising since the α-subunit contains the active site of the sMMO hydroxylase enzyme (Prior and Dalton, 1985a). Indeed the proposed iron binding motifs (Glu-X-X-His) are well

conserved in those bacteria in which the full cluster has been sequenced (Shigematsu *et al.*, 1999).

Table 1.3 Matrix comparing identity of DNA and derived amino acid sequences of *mmo* genes. Amino acid identities are shown in boldface type. KSPIII: *Methylomonas* sp. KSPIII; KSWIII: *Methylomonas* sp. KSWIII; *Mc* Bath: *Methylococcus capsulatus* Bath: *Ms* OB3b: *Methylosinus trichosporium* OB3b; *Mcy* str M: *Methylocystis* sp. strain M. Adapted from Shigematsu *et al.*, (1999).

Sequence			**% Identity**		
	KSPIII	**KSWIII**	***Mc* Bath**	***Ms* OB3b**	***Mcy* strain M**
***mmoX*/MmoX**					
KSPIII		**100**	**87**	**79**	**80**
KSWIII	97		**87**	**79**	**80**
Mc Bath	79	79		**82**	**82**
Ms OB3b	72	72	76		**96**
Mcy str M	72	72	77	92	
***mmoY*/MmoY**					
KSPIII		**100**	**56**	**57**	**61**
KSWIII	97		**56**	**57**	**61**
Mc Bath	62	62		**54**	**56**
Ms OB3b	61	61	67		**89**
Mcy str M	61	61	67	91	
***mmoZ*/MmoZ**					
KSPIII		**100**	**53**	**50**	**50**
KSWIII	98		**53**	**50**	**50**
Mc Bath	60	61		**49**	**51**
Ms OB3b	57	57	63		**87**
Mcy str M	58	58	64	87	
***mmoB*/MmoB**					
KSPIII		**100**	**70**	**70**	**70**
KSWIII	99		**70**	**70**	**70**
Mc Bath	69	69		**66**	**66**
Ms OB3b	67	67	67		**96**
Mcy str M	68	68	68	95	
***OrfY*/OrfY**					
KSPIII		**100**	**38**	**35**	**38**
KSWIII	98		**38**	**35**	**38**
Mc Bath	53	52		**40**	**37**
Ms OB3b	55	54	54		**59**
Mcy str M	58	57	55	74	
***mmoC*/MmoC**					
KSPIII		**99.7**	**61**	**46**	**49**
KSWIII	98		**59**	**48**	**50**
Mc Bath	62	61		**49**	**51**
Ms OB3b	55	56	59		**81**
Mcy str M	54	55	61	81	

The reductase enzyme is believed to contain a [2Fe-2S] cluster, a hypothesis which is supported by the finding that the derived amino acid sequence of the *mmoC* gene contains the four conserved cysteine residues common among [2Fe-2S] clusters of ferredoxins and has significant identity with ferredoxins from plants and bacteria (Cardy *et al.*, 1991; Stainthorpe *et al.*, 1990b). As mentioned previously the sMMO enzyme possesses a small regulatory protein, protein B, encoded by the *mmoB* gene. The deduced amino acid sequences of protein B from *Methylosinus trichosporium* OB3b has 28% identity with the corresponding proteins involved with the toluene/benzene-2-monooxygenase (TbmC) from *Pseudomonas* sp. strain JS150 (Johnson and Olsen, 1995), (P2 component of) phenol hydroxylase from *Pseudomonas* sp. strain CF600 (Qian *et al.*, 1997) and the alkene monooxygenase (AMO) of *Rhodococcus rhodocrous* B276 (formerly *Nocardia corallina* B276) (Saeki and Furhashi, 1994).

1.4.3 Heterologous expression of sMMO

Expression of sMMO in heterologous hosts has met with mixed success over the years. The genes *mmoB* and *mmoC*, encoding the coupling protein B and the reductase component (protein C) from *Methylococcus capsulatus* Bath, have been functionally expressed in *E. coli* using a T7 polymerase expression system (West *et al.*, 1992). However, attempts to express the hydroxylase were not successful, possibly due to the inability of *E. coli* to correctly fold the sMMO enzyme complex (West *et al.*, 1992). Attempts to express the hydroxylase component of *M. trichosporium* OB3b in *E. coli* also resulted in inactive preparations (Jahng *et al.*, 1996). Functional expression of the *M. trichosporium* OB3b sMMO complex has been reported in *Pseudomonas putida* F1 (Jahng and Wood, 1994), but attempts by Lloyd (1997) to repeat this expression were unsuccessful.

The first successful demonstration of heterologous expression of sMMO from *M. trichosporium* OB3b was provided by Lloyd *et al.*, (1999b). They expressed the sMMO cluster on a broad-host range plasmid (pVK100Sc) containing the native *mmoX* promoter in the pMMO-only methanotrophs *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP (Lloyd *et al.*, 1999b). Presumably, these organisms possessed the proteins necessary for the correct assembly of the sMMO-hydroxylase. Interestingly, expression of sMMO in *Methylomicrobium album* BG8 was only present at low copper-to-biomass ratios. Thus, it appears that *Methylomicrobium*

album BG8 contains the regulatory system for sMMO or that the regulatory sequences/polypeptides controlling sMMO expression were present on the expression plasmid.

Lloyd *et al.*, 1999a also achieved expression of the sMMO enzyme in an *mmoX*⁻ mutant of *Methylosinus trichosporium* OB3b using the plasmid pVK100Sc, causing the recovery of sMMO activity. This plasmid is believed to be stably maintained in *M. trichosporium* OB3b and results in a strain that still expressed the sMMO polypeptides at copper concentrations up to 7.5 μM (Normally, sMMO expression is lost at copper concentrations of 0.25 μM -1 μM) (Lloyd *et al.*, 1999a). The expression of the sMMO operon at elevated copper concentrations was possibly due to the increased copy number of sMMO genes carried on pVK100Sc, increasing the threshold level of copper required to repress sMMO transcription.

1.5 Transcriptional regulation of the *pmo* and *mmo* operons

1.5.1 "The copper switch"

In methanotrophs possessing both the soluble and particulate methane monooxygenase enzymes a unique metabolic switch mediated by copper ions occurs. At low copper- to- biomass ratios (less than 0.89 μmol copper per g [dry weight]) (Hanson & Hanson, 1996) the soluble form of the enzyme is expressed, whilst at high copper-to-biomass ratios it is the particulate methane monooxygenase that is expressed. Stanley *et al.*, first observed this phenomenon in 1983. They reported that the cellular location of methane monooxygenase activity in chemostat cultures of *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b grown on low copper NMS medium (of Whittenbury *et al.*, 1970) switched from the membrane to soluble fractions with increasing biomass levels (above 0.8 mgl^{-1}), but that when grown in high copper medium (supplemented with 1 mgl^{-1} $CuSO_4.5H_2O$) the biomass level at which this switch occurred approximately doubled (1.6 mgl^{-1}). The switch in location of methane monooxygenase activity was accompanied by the appearance of extensive intracytoplasmic membrane structures believed to harbour the pMMO enzyme (Scott *et al.*, 1981, Dalton *et al.*, 1984). In addition to the change in location of MMO activity, this switch can be followed by SDS-PAGE. The switch to sMMO expression is accompanied by the appearance of large amounts of the α, β, and γ subunits of the sMMO-hydroxylase which can be observed by Coomassie blue

staining (Stanley *et al.*, 1983; Figure 7.2). It is also possible to observe the 45 kDa PmoC protein by the same method (Zahn and Dispirito, 1996).

However, not until the work of Nielsen *et al.*, (1996, 1997) was a detailed analysis of *mmo* and *pmo* transcription performed. These workers identified the transcriptional start sites for the *mmo* clusters from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath by primer extension (Figure 1.6). The promoter identified 5' of the transcriptional start site in *Methylosinus trichosporium* OB3b differs by only one base from the consensus for the σ^N-type promoter (TGGCAC-N_5-TTGCA) (Merrick, 1993) at the most variable position in the consensus sequence. An alignment of this sequence with the corresponding regions 5' of *mmoX* from *Methylomonas* sp. KSW III and *Methylocystis* sp. strain M show that these organisms also contain putative σ^N promoters (Figure 1.6). However, the promoter found 5' of the transcriptional start site identified for the *mmo* cluster from *Methylococcus capsulatus* Bath has a –24 motif but only matches the highly conserved –12 motif at 3/6 bases (Nielsen *et al.*, 1996) and was suggested to be non-functional since it lacks the highly conserved GC pair (Nielsen *et al.*, 1997; Barrios *et al.*, 1999). It therefore seems that with the possible exception of the *mmo* operon from *Methylococcus capsulatus* Bath, these genes seem to be transcribed from σ^N-type promoters 5' of *mmoX* in the methanotrophs shown in Figure 1.6. One of the aims of the research presented in this thesis was to establish whether transcription of the *mmo* cluster from *Methylosinus trichosporium* OB3b and *Methylococus capsulatus* Bath is dependent on the gene product of the *rpoN* gene, σ^N (or σ^{54}), and these data are presented in Chapter 5. Transcriptional regulation involving σ^N is reviewed in section 1.7.

```
                -24          -12              +1
Ms OB3b         CCGCAGCGAGTGGCACAGGCCTTGCCAAATAAGTCGACGCTTCC
Mcy strain M    TCACGATGACTGGCACGCGCCTTGCCAAATAAGTCGGGTCATCG
Mm KSW III      AACGCAATACTGGCACACGTGTTGCAATCTGACCACCGAGGCTG
Mc Bath         TACGATAAAGTGGCACGATCCCTGTAACTAGGTTGTCACGACCT
Consensus                 TGGCAC- N5-TTGCA
```

Figure 1.6 Alignment of putative σ^N promoter regions of four *mmo* clusters.
The transcriptional start sites (highlighted in blue) for *Methylosinus trichosporium* OB3b (*Ms*OB3b) and *Methylococcus capsulatus* Bath (*Mc* Bath) were mapped by primer extension (Nielsen *et al.*, 1996, 1997). For *Methylocystis* sp. strain M (*Mcy* strain M) and *Methylomonas* sp. KSW III (*Mm* KSW III) hypothetical start sites are indicated. Only those nucleotides matching the σ^{54} promoter consensus (Barrios *et al.*, 1999) are highlighted in bold text.

Further primer extension experiments by Nielsen *et al.*, (1997, 1996) revealed a second transcriptional start site in the intergenic region between *mmoX* and *mmoY* of *Methylosinus trichosporium* OB3b at A_{2161}. Upstream (5') of this position is a putative σ^{70}-type sequence ($_{2126}$TTCCCC-N_{17}-TCTGAT$_{2153}$). An alignment between the *mmoX-mmoY* intergenic regions of *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M reveals that this putative σ^{70} promoter sequence is present in both organisms (Figure 1.7).

A

```
                TTGACA   - N17 -      TATAAT -N7- N
        2115
Ob3b    CTGCGTGTTCCCCGATCCGCTCGCCAAATTCTGATCTCACGAGATTCCGGCGCGCGTCGCTCGCAACGCGCGTCGGATGAA
M       CTGCGTCTTCCCCGATCCGCTCGCGAAATTCTGATCTTCATTCGCTTCGCGTCGAATTGCAACGGGTTCCCGGCGCGTTTC
        ************************************        * ***  **  * **        * ** **  *
        2206

OB3b    CCGCCGGGCCTCCCGCGTCCGGCGTCTCCTTCGACGCGAGCACGAAGCACCAAAAATCCTTCTGTGGAGGAAGAACTCACC
M       GCGCCGGGTTCTCGGCGGGCGACGTGCGTCCGTCGACAAAGCACCAAGCACGAAAATCCCCTAGTGGAGGACATCTGGA--
         *******    * ***  ** ***           * *      *    * *******    ********
```

B

```
                TTGACA   - N17 -      TATAAT -N7- N
        3771
KSW     CTGCGTGTTTTCCGATCCTTTGAAAGCCCTTTAATCGTTTCAATTCTTTAAGGAAATAGCTT
```

Figure 1.7 Putative promoter sequences located in intergenic region between *mmoX* and *mmoY*.
A: Alignment of intergenic region from *Methylosinus trichosporium* OB3b (OB3b) and *Methylocystis* sp. strain M. The nucleotide start position of the alignment is numbered according to Accession numbers: X55394 and U81594 respectively. The putative σ^{70} promoters and transcriptional start sites as identified by Nielsen *et al.*, are shown in bold type for *M. trichosporium* OB3b, and the corresponding motifs in *Methylocystis* sp. strain M were predicted based on this sequence. Ribosome binding site for *mmoY* is highlighted in red and the 3' end of *mmoX* is underlined. Nucleotide positions identical in both sequences are denoted by asterisks. Gaps were introduced to improve alignment.
B: Intergenic region between *mmoX* and *mmoY* from *Methylomonas* sp. strain KSW III (KSW) showing putative σ^{70} promoter (bold type) and predicted transcriptional start site. The 3' end of *mmoX* is underlined and the ribosome binding site for *mmoY* is shown in red type.
In all cases, the ATG codon of *mmoY* immediately follows the last nucleotide shown.

Northern analysis performed by the same researchers revealed three mRNA molecules: mRNA-1 (1.8 kb) originated 5' of *mmoX* and is believed to originate from $P\sigma^N$, encoding *mmoX* only; mRNA-2 (4.0 kb) which covers *mmoYBZorfYmmoC* originated 5' of *mmoY;* and mRNA-3 (2.2 kb) covering *mmoYBZ* also originating 5' of *mmoY*. These data would appear to suggest that the *mmo* operon of *Methylosinus trichosporium* OB3b is transcribed from a σ^N promoter 5' of *mmoX* giving rise to the 1.8 kb mRNA-1 and from the σ^{70} promoter between *mmoX* and *mmoY* giving rise to mRNA-2 and mRNA-3 encoding the rest of the *mmo* genes as polycistronic mRNA molecules. Transcription from both promoters appears to be regulated in response to copper ions since primer extension and Northern analysis performed on RNA extracted from low-copper grown cells showed the loss of these transcripts 30 min after the addition of 50 μM copper to steady state chemostat cultures. This hypothesis seems to be supported by the alignments shown in Figure 1.7, and also indicates that this phenomenon may occur in *Methylocystis* sp. strain M and possibly in *Methylomonas* sp. strain KSW III. However, it is possible that this primer extension signal arose from the processing of a longer, unstable mRNA molecule encoding all the *mmo* genes and originating from the σ^N promoter 5' of *mmoX* in *Methylosinus trichosporium* OB3b.

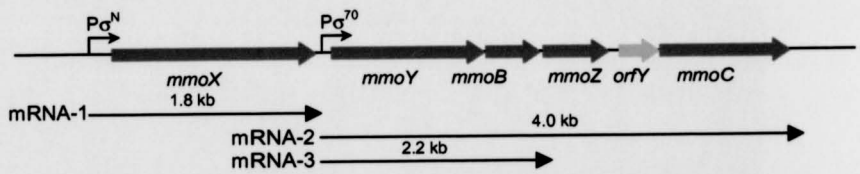


Figure 1.8 *mmo* gene cluster from *Methylosinus trichosporium* OB3b indicating proposed location of putative promoter regions and mRNA transcripts (adapted from Nielsen *et al.*, 1997).

This story is further confused by the findings of Northern analysis and primer extension carried out on the *mmo* cluster from *Methylococcus capsulatus* Bath (Nielsen *et al.,* 1996, 1997). No putative transcriptional start sites were identified in the region between *mmoX* and *mmoY*. In addition, Northern analysis revealed 3 transcripts all originating from the promoter 5' of *mmoX* (Figure 1.9). Whilst it is clear that only one promoter is responsible for *mmo* transcription in *Methylococcus capsulatus* Bath, it is not clear whether this cluster is transcribed from a σ^N or

σ^{70}-type promoter. Thus, the role of the *mmoX-mmoY* intergenic promoter in *Methylosinus trichosporium* OB3b remains elusive.

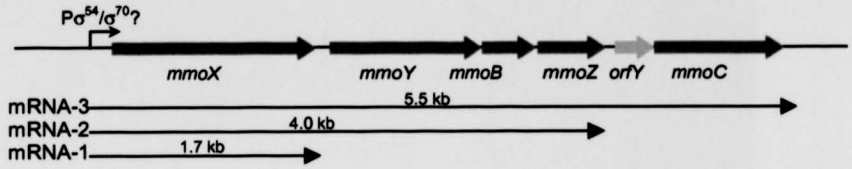


Figure 1.9 *mmo* gene cluster from *Methylococcus capsulatus* Bath indicating proposed location of putative promoter regions and mRNA transcripts (adapted from Nielsen *et al.*, 1997).

Although some of the finer details of this transcriptional regulation are uncertain it is clear that they are dramatically affected by the addition of copper to the growth medium. Nielsen *et al.*, (1997, 1996) showed that *mmo* specific primer extension and Northern hybridisation signals disappeared within 30 minutes of the addition of copper (final concentration of 50μM) to the growth media of *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b. At the same time, induction of *pmo*-specific transcription in *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath occurs, as analysed by Northern analysis (Nielsen *et al.*, 1997). Thus copper ions cause a switch between transcription of the *mmo* and *pmo* operons- "the copper switch".

Transcription of the *pmo* operons has been studied in some detail in *Methylococcus capsulatus* Bath. As mentioned in section 1.3.2, this organism contains two complete copies of the *pmoCAB* operon and a third copy of the *pmoC* gene (Stolyar *et al.*, 1999). Stolyar *et al.*, (2001) used primer extension to identify putative transcriptional start sites 5' of *pmoC* for both copies of the *pmo* operon from *Methylococcus capsulatus* Bath which mapped with putative σ^{70} promoters (Figure 1.10). However, no transcriptional start sites were identified between *pmoC-pmoA* or *pmoA-pmoB*, which agreed with the finding by Nielsen *et al.*, (1997) that the *pmoCAB* operon is transcribed as a 3.3 kb polycistronic RNA molecule which is processed into several smaller molecules. These workers also identified a conserved sequence motif overlapping the –35 element of this promoter which is a proposed regulatory motif (Figure 1.10). In order to examine the expression levels of the two copies of the *pmoCAB* cluster in response to varying copper levels, *pmo* copy-specific *xylE*-transcriptional fusions were constructed (Stolyar *et al.*, 2001). These data

revealed that copy 2 was expressed at higher levels than copy 1 at copper concentrations up to 5μM, but at higher copper concentrations (>50μM) both copies were expressed to approximately the same degree (Stolyar *et al.*, 2001). Interestingly *pmo* copy 2 transcripts were detected even at low copper concentrations using both *xylE*-transcriptional fusions and Northern analysis. Thus, the function of the multiple *pmoCAB* copies in methanotrophs is still unclear. It is clear that under laboratory conditions, one copy of *pmoCAB* can compensate for the absence of the other, but that the role of both copies of *pmoCAB* in nature is unknown. Until recently, only the *pmo* operon from *Methylococcus capsulatus* Bath had been sequenced. Gilbert *et al.*, (2000) reported the sequence of one complete copy of the *pmoCAB* operon from *Methylocystis* sp. strain M and *Methylosinus trichosporium* OB3b. The identification of transcriptional start sites and putative promoters and a comparison with those found in *Methylococcus capsulatus* Bath is presented in Chapter 3 of this thesis.

```
                    >>>>>       <<<<<
Copy 1  CTACCCCTGCGTCAAAATGTCGCAGATTTTTCTTGACAGTTTGGGGGAGGGTGATAGACTCCCTCCA
Copy 2  CTGAGCCTGCGTCAAAATGACGCATATTTTTCTTGACAGCCTCGGGTTGGGTGATAGACTGCGACCCA
        ************************************  * ***  ************ *  **
Consensus                                   TTGACA      -N17-      TATAAT
                                             -35                    -10
```

Figure 1.10 Alignment of conserved regions 5' of *pmoC* in *Methylococcus capsulatus* Bath. The promoter and transcriptional start sites are shown in bold. Conserved residues are denoted by an asterisk and the proposed regulatory inverted repeats shown with arrowheads.

A model for the regulation of the *mmo* and *pmo* operons in response to copper was proposed by Nielsen *et al.*, (1997) and is shown in Figure 1.11. A further aim of this thesis was to try to verify a role for σ^N and to identify the proposed regulator, which interacts with σ^N at $P\sigma^N$. In light of the results presented in this thesis an improved model for this system is presented in Chapter 8.

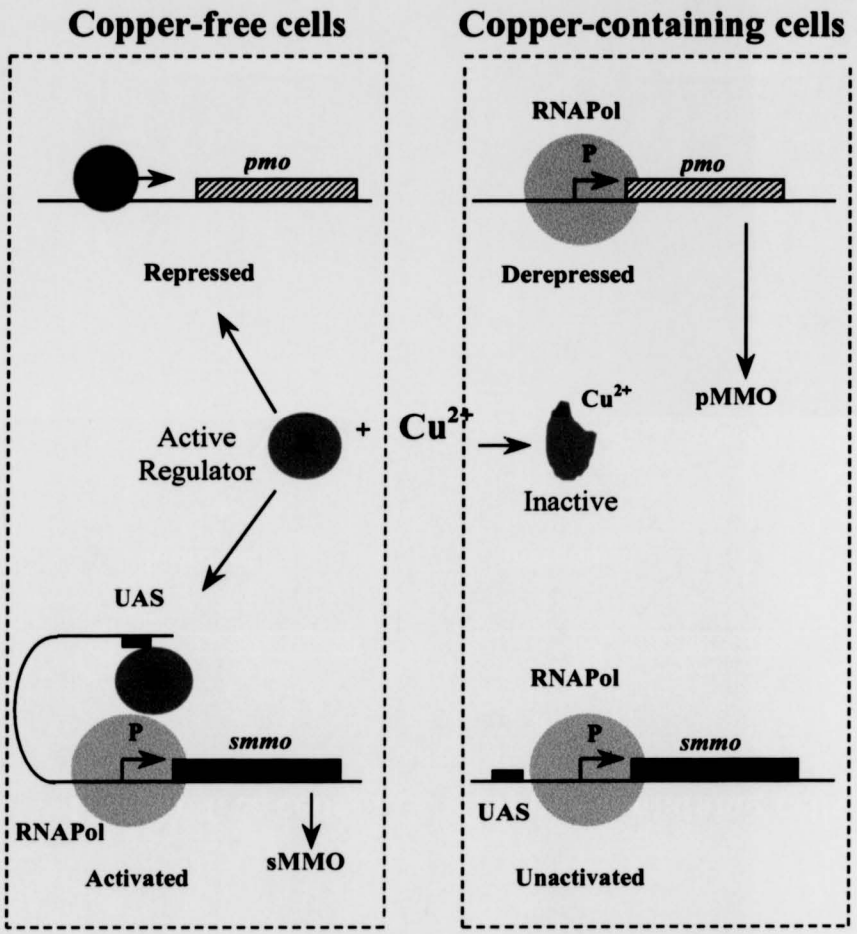


Figure 1.11 Hypothetical model for regulation of methane monooxygenase genes. (from Stanley *et al.*, 1997). In 'copper-free' cells a regulator protein binds to the *pmo* operon and represses transcription. The same (or another) regulator binds at an upstream activator sequence (UAS) 5' of the *mmoX* σ^N promoter and makes contact with σ^N-containing RNA polymerase, which induces transcription of the *mmo* genes. In 'copper-excess' cells, copper inactivates the regulator protein causing derepression of *pmo* transcription and inactivation of *mmo* transcription.

1.5.2 Expression of sMMO and pMMO from methanol grown cells

The first report of methane monooxygenase expression in methanol grown cells of *Methylosinus trichosporium* OB3b was published by Best and Higgins (1981). In subsequent years several studies have showed that methanol-grown cells possess MMO activity but have disagreed on the cellular location of this activity, i.e. pMMO versus sMMO (Best and Higgins, 1981; Davis *et al.,* 1987; Prior and Dalton, 1985b). However, the work of Finch (PhD thesis, 1995) represents the most recent and complete study of sMMO and pMMO expression in methanol grown cells. Finch (1997) showed that chemostat cultures of *Methylosinus trichosporium* OB3b grown in the presence of low concentrations of copper (where sMMO and not pMMO was expected to be present) possessed the pMMO enzyme when grown on limiting (50mM) and excess (200mM) concentrations of methanol. However, this was present as a chiefly inactive pool of pMMO. The cell-free extracts from cultures grown with a limiting concentration of methanol had an undetectable level of pMMO activity that increased to 20 nmol/min/mg of protein on the addition of $CuSO_4$ to a final concentration of 0.4 mM. Similarly, pMMO activity of cell free extracts from cells grown with excess methanol increased from 38 nmol/min/mg of protein to 74 nmol/min/mg of protein when the same concentration of copper was added (0.4 mM).

While it is clear that pMMO is expressed in cells grown on methanol, the situation regarding sMMO is less clear. In the presence of excess methane and limiting methanol (50 mM), chemostat cultures of *M. trichosporium* OB3b expressed low levels of sMMO activity and polypeptides (as assessed by Western blotting). When these cultures were switched to limiting methanol concentrations (methane switched off) the sMMO activity disappeared, but Western blots were inconclusive and showed the possible presence of sMMO-hydroxylase subunits. Similar results were obtained in the presence of excess (200mM) methanol. Thus, it appears that sMMO expression is inhibited by methanol. Therefore, a further aim of this thesis was to assess at a qualitative level the presence of sMMO and pMMO transcripts in methanol grown cells of *M. trichosporium* OB3b.

1.6 Gene inactivation in methanotrophs

The first example of gene-specific inactivation in methanotrophs was provided by Toukdarian and Lidstrom (1984a), who demonstrated marker-exchange mutagenesis of *nif*-specific genes in the obligate methanotroph *Methylosinus* sp. strain 6. By this method they succeeded in the transfer of a Tn5-Km marker into the chromosome of this organism. A similar strategy was employed by Martin and Murrell (1995) to construct a stable mutation in the *mmoX* gene of *Methylosinus trichosporium* OB3b (Figure 1.12). The *mmo* cluster from *M. trichosporium* OB3b was ligated into pBR325, which does not replicate in methanotrophs, before a 1.2 kb *Xho*I restriction fragment was excised from the *mmoX* gene and replaced by a kanamycn resistance gene. The RP4-*mob* cassette was then ligated into this vector to create pHM32m (Figure 1.12). This vector was transferred into *M. trichosporium* OB3b by conjugation from *E.coli* S17-1, which carries a chromosomal copy of the RP4 transfer functions, allowing conjugal transfer of plasmids containing the RP4-*mob* cassette (Simon *et al.*, 1989). Since pHM32m cannot replicate in *M. trichosporium* OB3b, kanamycin (Km) resistant colonies only arose as a result of a homologous recombination events between the *mmoX* gene on pHM32m and in the chromosome of *M. trichosporium* OB3b (Figure 1.12). In these experiments eight Km-resistant colonies were isolated at a frequency of 5×10^{-8} per recipient. Analysis of sMMO-expression in the Km-resistant colonies was analysed by the naphthalene assay (Section 2.23) (Brusseau *et al.*, 1990) and showed that six of these strains did not oxidise naphthalene, indicating an $sMMO^-$ phenotype. Analysis of these mutants by Southern-hybridisation showed that five of the $sMMO^-$ strains had undergone a double-recombination event. Whereas one strain contained two copies of the *mmoX* gene in its chromosome, indicating a single-recombination event had occurred, leaving the whole of pHM32m in the chromosome. Stolyar *et al.*, (1999) have also shown that this method can be successfully employed in *Methylococcus capsulatus* Bath, by mutation of both copies of the *pmo* genes from this organism. Thus, it is possible to inactivate specific genes in methanotrophs to create stable mutants by marker-exchange mutagenesis, albeit at low frequencies.

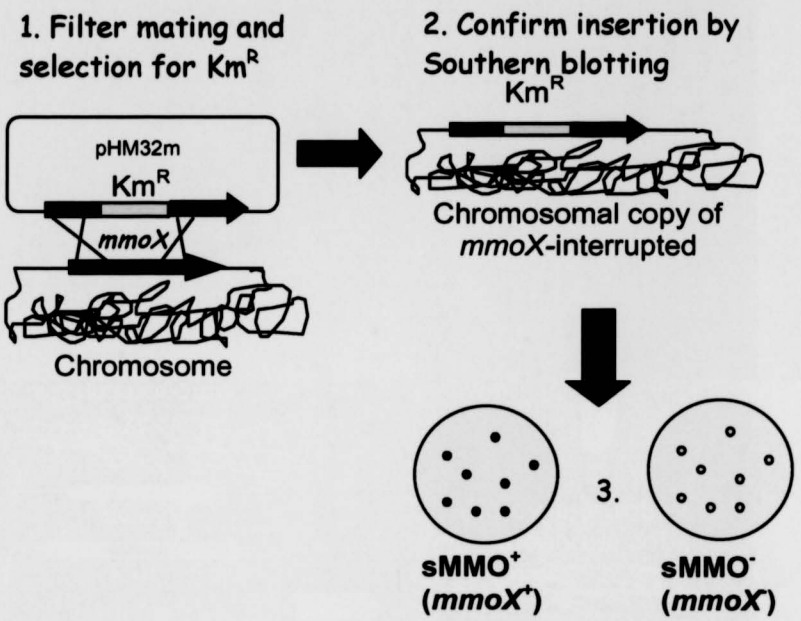


Figure 1.12 Schematic representation of Marker-exchange mutagenesis of *mmoX* gene by Martin and Murrell (1995).
1. A suicide vector containing an interrupted version of *mmoX* and the RP4-*mob* cassette was introduced into *M. trichosporium* OB3b by conjugation from *E.coli* S17-1, exconjugants were selected with kanamycin (10 $\mu g ml^{-1}$). 2. Homologous recombination between plasmid and chromosomal copies of *mmoX* results in insertion of the Km^R cassette into the chromosomal copy of *mmoX*. Confirmed by Southern blotting. 3. The naphthalene assay was used to detect sMMO activity on low copper plates. strains containing wild-type *mmoX* gene oxidised naphthalene and colonies appeared purple. strains containing inactivated *mmoX* gene remained orange.

The recombination frequencies observed by Martin and Murrell (1995) were similar to those achieved by Martin and Murell (1995) and Finch (1997) for the introduction of pDSK509ΩSc (2.8×10^{-8}) and pVK100Sc (1×10^{-8}), both of which contained the whole *mmo* gene cluster in a broad-host range cloning vector. These data indicated that whilst transformation frequencies for *Methylosinus trichosporium* OB3b are relatively low, the frequency at which Martin & Murrell (1995) gained an *mmoX* mutant was similar to that achieved for the transfer of broad-host-range plasmids, suggesting that homologous recombination occurs at a high frequency in this organism.

1.7 Regulation by the σ^N sigma subunit of RNA polymerase

As mentioned above, transcription of the *mmo* operon of several methanotrophs is thought to proceed from a σ^N-type promoter located 5' of the *mmoX* gene. Thus, it is likely that the alternative sigma factor σ^N (or σ^{54}) is involved in the initiation of transcription of these genes.

1.7.1 σ^N: the *rpoN* gene product

The alternative sigma factor σ^N (or σ^{54}) is encoded by the *rpoN* gene (rna polymerase N), denoted σ^N due to its intimate involvement with nitrogen metabolism. Indeed, it was originally discovered as a gene required for the synthesis of glutamine synthetase in *Salmonella* (Garcia *et al.*, 1977) and named *glnF*, it has also been known as *ntrA* (Merrick & Gibbins, 1985; Merrick & Stewart, 1985). However, since that time it has been shown to be involved in the transcriptional regulation of a wide variety of genes (see Table 6.1) (reviewed in Merrick, 1993; Shingler, 1996; Studholme and Buck, 2000a,b). There appears to be no common theme in the range of processes controlled by σ^N except that they are non-essential cellular processes, such as nitrogen fixation or toluene degradation.

The first *rpoN* genes to be discovered were chiefly from enteric bacteria, but with the increasing number of bacterial genome sequences available it is becoming clear that many genera possess *rpoN* (Studholme and Buck, 2000a,b). A recent survey showed that *rpoN* is present in 35 organisms whose genomes have been at least partially sequenced, including organisms as diverse as *Bacillus subtilis*, *Aquifex aelicolus*, *Sinorhizobium meliloti*, and *Planctomyces limnophilus* (Studholme and Buck, 2000b). In many of these organisms *rpoN*, is present in a single copy in the genome in a locus with several linked genes (see section 5.2.4) (Merrick, 1993; Powell *et al.*, 1998). However, *Bradyrhizobium japonicum*, *Rhizobium etli* and *Rhodobacter sphaeroides* contain two different *rpoN* genes (Wosten *et al.*, 1998). In most cases the *rpoN* gene is constitutively expressed, but in some cases it is subject to transcriptional regulation. In *Bradyrhizobium japonicum* the *rpoN2* gene is negatively autoregulated (Kullik *et al.*, 1991). Negative autoregulation has also been observed for the *rpoN1* gene from *Rhizobium etli* under aerobic conditions, but its expression is reduced under microaerobic conditions where *rpoN2* is expressed (Michiels *et al.*, 1998a).

In contrast to σ^{70}-type sigma factors the promoter sequences to which σ^N is able to bind are very well conserved and relatively invariant (Wösten, 1998). An alignment of 186 σ^N promoters by Barrios *et al.*, (1999) confirmed the consensus promoter for recognition by σ^N as TGGCAC-N_5-TTGC(A/T)-N_7-N. The highlighted GG (-24) and GC (-12) motifs are present in 99.5% and 95.5 % of the 186 promoters analysed, which is not surprising considering that they have been shown to contact σ^N directly (Barrios *et al.*, 1999).

In addition to having a highly conserved promoter recognition element, the σ^N protein, RpoN, has a highly conserved domain organisation (Figure 1.13) (reviewed in Buck *et al.*, 2000; Merrick *et al.*, 1993; Wösten *et al.*, 1998). There are three main regions: Region I is proposed to respond to activation signals and inhibit RNA polymerase isomerisation; Region II is highly variable across all known σ^N proteins; and the third region is known to be important in DNA-binding (Buck *et al.*, 2000). Binding of the protein to the core polymerase is believed to involve parts of both region I and III.

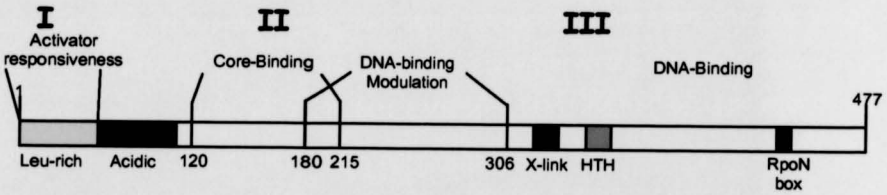


Figure 1.13 Domain organisation of the σ^N protein from *Escherichia coli* (from Buck *et al.*, 2000). σ^N can be divided into 3 domains according to Merrick (1993): I, involved with activator responsiveness; II, Acidic variable region; III, DNA-binding. Amino acids 120-215 is involved with binding core RNA polymerase protein. The region from amino acids 180-306 is proposed to modulate DNA-binding. RpoN may directly interact with the -24 consensus sequence, the helix-turn-helix (HTH) motif and cross-linking region may also interact with DNA.

Although the range of genes over which σ^N proteins acts is large, the mechanism by which it is believed to activate transcription may explain the broad nature of this range. The σ^N protein is capable of binding to both the promoter element and RNA-polymerase to form a stable σ^N containing holoenzyme complex. It is proposed that the function of σ^N is to lock this complex into a closed conformation, preventing initiation of transcription in the absence of the specific environmental stimulus (Buck *et al.*, 2000). Deregulated transcription observed following mutations in regions I and III (Chaney *et al.*, 1999) and in the –12 region of the promoter element (Wang and Gralla, 1998) seem to support the idea of a complex

interaction between region I and III and the –12 promoter region, forming a stable closed complex (Buck *et al.*, 2000). Initiation of transcription requires the action of an activator protein which interacts with σ^N, possibly via region I and causes a conformational change resulting in the formation of an open-complex by a mechanism requiring ATP. In this way, σ^N binds to its promoter elements and is activated by an activator specific for the gene in front of which the promoter lies. Transcriptional activation by σ^N does not proceed in the absence of an activator protein, and it is these proteins that form the subject area of section 1.7.2.

1.7.2 σ^N-dependent activators- Enhancer Binding Proteins

The σ^N protein is not capable of forming an open transcriptional complex in the absence of a transcriptional activator protein (Merrick, 1993). The family of proteins involved in initiation of transcription via σ^N-containing RNA polymerase ($E\sigma^N$) are termed enhancer-binding proteins (EBPs) (see table 6.1). These activators are known as EBPs due to the fact that they exert their control over $E\sigma^N$ by binding at distant enhancer sequences (Morrett and Segovia, 1993). These enhancer binding sites are palindromic, or nearly palindromic, nucleotide sequences normally found 100-200 bp 5' of the $E\sigma^N$ promoter, but are still able to stimulate transcription from $E\sigma^N$ promoters if moved thousands of nucleotides away (Reitzer *et al.*, 2001; Morrett and Segovia, 1993). The mechanism by which the EBPs are able to cause transcriptional activation from distant sites requires direct contact between the EBP and $E\sigma^N$. This is possible due to bending of the DNA between the EBP binding site and $E\sigma^N$ bound at the promoter (Carmona *et al.*, 1997; Hoover *et al.*, 1990). In many cases this bending of DNA is facilitated by the binding of the integration host factor (IHF), at a site between the EBP binding site and the σ^N promoter element (Hoover *et al.*, 1990). The binding site for IHF is split into two regions, a 3' conserved region with the consensus sequence: WATCNNNNTTR (Goodman *et al.*, 1999; Goodrich *et al.*, 1990; Hoover *et al.*, 1990) and a degenerate 5' region characterised by an unusually high frequency of A and T residues (Goodman *et al.*, 1999). In some cases, such as NtrC stimulated activation of the *nifLA* operon of *Klebsiella pneumoniae*, IHF is not involved and bending of DNA is caused by intrinsic curvature due to tracts of A and T residues (Cheema *et al.*, 1999; Carmona *et al.*, 1997). After the binding of an EBP at its enhancer region and the bending of intervening DNA to allow direct

contact between the EBP and $E\sigma^N$, the next step in activation is an EBP catalysed conformational change in the $E\sigma^N$-promoter complex which causes open complex formation (North *et al.*, 1993). This process requires energy gained from the hydrolysis of an ATP molecule by the EBP (Porter *et al.*, 1995; North *et al.*, 1993).

The primary structure of the EBPs reveals a striking modularity between all members of this family (reviewed by Morrett and Segovia, 1993). They are comprised of a highly conserved central domain containing ATP-binding motifs, a C-terminal DNA-binding domain containing a characteristic helix-turn-helix motif and a highly variable N-terminal domain (Figure 1.14) (Morrett and Segovia, 1993; Porter *et al.*, 1995). An amino acid alignment and description of the signature motifs of the central and C-terminal domains of 10 EBPs can be found in Chapter 6 (Figure 6.2).

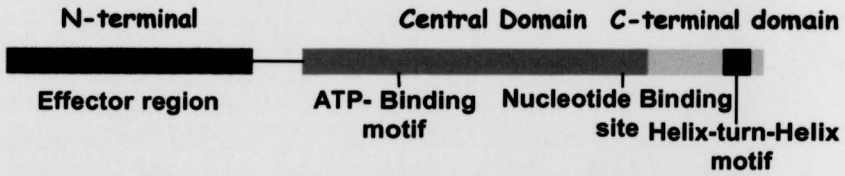


Figure 1.14 Modular structure of Enhancer Binding Protein transcriptional activators.

As mentioned in section 1.6.1, $E\sigma^N$ acts in the initiation of transcription of a diverse range of functional gene clusters (for examples see table 6.1). It is the conserved mechanism of action and modular nature of the EBPs and σ^N, which allows this to occur. As discussed in section 6.6 it is the N-terminal domain of these proteins which confers effector specificity via a number of mechanisms, thus allowing the same family of proteins to be able to respond to several types of signal and exert effects over a diverse range of genes.

1.8 Nitrogen metabolism in methanotrophs

1.8.1 Nitrogen fixation

The first detailed study of nitrogen fixation in methanotrophs was conducted by Murrell and Dalton (1983a) and revealed nitrogenase activity in the type II methanotroph *Methylosinus trichosporium* OB3b and the type I methanotroph *Methylococcus capsulatus* Bath using the acetylene reduction assay (Dalton and Whittenbury, 1976). However, nitrogenase activity was not detectable in type I methanotrophs, including *Methylomicrobium album* BG8 or *Methylomonas* sp. S1 (Murrell and Dalton, 1983a).

The nitrogenase activity from *M. trichosporium* OB3b was unusual in that it was remarkably aerotolerant (Murrell and Dalton, 1983a). This was also observed by Toukdarian and Lidstrom (1984a) in the strain *Methylosinus sporium* sp. 6. In contrast, the nitrogenase from *Methylococcus capsulatus* Bath is much less aero-tolerant (2-fold) (Murrell and Dalton, 1983a).

Until the work of Oakley and Murrell (1988), it was thought that only type II methanotrophs and *Methylococcus capsulatus* Bath possessed a nitrogenase enzyme. However, heterologous probing using the *nifH* gene (encoding the Fe-containing subunit of nitrogenase) from *Klebsiella pneumoniae* revealed that several type I and type II methanotrophs possessed putative *nifH* homologues (Murrell, 1992). This finding was recently confirmed by the detection of *nifH*-specific PCR products and nitrogenase activity from several species of *Methylocystis* (type II), *Methylosinus* (Type II) and *Methylomonas* (Type I) (Auman *et al.*, 2001). It is therefore becoming clear that many methanotrophs possess nitrogenase enzymes, an observation supported by the finding of several *nifH*-specific DNA sequences amplified by PCR from a variety of environmental sources (Auman *et al.*, 2001).

The structural genes encoding the nitrogenase enzyme have only been cloned from two methanotrophs (Oakley and Murrell, unpublished; Toukdarin and Lidstrom, 1984b). The nitrogenase enzyme in the case of *Klebsiella pneumoniae* (Brock *et al.*,1994) is encoded by a large gene cluster of at least 20 *nif* genes, comprising of the structural genes for the nitrogenase itself (*nifHDK*) and several proteins enabling insertion of cofactors and assembly. Toukdarian and Lidstrom (1984b) reported the cloning of *nif* specific DNA and the creation of a *nif*$^-$ strain of *Methyosinus sporium* sp. 6 by marker-exchange mutagenesis in what was likely to be the *nifD* gene. The

nifHDK genes have been cloned and sequenced from *Methylococcus capsulatus* Bath (Murrell and Oakley, unpublished data; reviewed in Murrell, 1992) and are similar to *nif* genes from other organisms, such as *Klebsiella pneumoniae*. The activity of nitrogenase in methanotrophs is known to be affected by two factors: fixed nitrogen levels and oxygen tension (Murrell and Dalton, 1983a; Toukdarian and Lidstrom, 1984a; Kim *et al.*, 2001). Nitrogenase activity is undetectable in methanotrophs grown in ammonia (AMS) or nitrate containing medium (NMS) (Murrell and Dalton, 1983a) and is known to decrease with increasing oxygen tension (Murrell and Dalton, 1983a; Kim *et al.*, 2001).

1.8.2 Ammonia and nitrate utilisation

Methanotrophs are capable of growth using nitrate as their sole nitrogen source in addition to nitrogen and ammonia. The use of nitrate as sole nitrogen source requires reduction to nitrite and then ammonia, which is catalysed by assimilatory nitrate reductase and nitrite reductase, respectively (Lin and Stewart, 1998). The product of both nitrate reduction and nitrogen fixation is ammonia, which is assimilated into cell biomass through one of two main pathways (Figure 1.15). Many organisms assimilate low concentrations of ammonia via the glutamine synthetase/ glutamate synthase (GS/GOGAT) pathway whilst utilising the alternative enzyme glutamate dehydrogenase (GDH) at higher ammonia levels (Merrick and Edwards, 1995) (Figure 1.15). In some organisms which do not possess GDH, ammonia present at high concentrations is assimilated via alanine dehydrogenase (ADH) which catalyses the synthesis of alanine from pyruvate and ammonia (Figure 1.15) (Murrell and Dalton, 1983b). Several other organisms possess only the GS/GOGAT pathway for ammonia assimilation, which they express constitutively. As discussed in section 5.6.2 this constitutive GS activity may comprise of up to four unrelated GS enzymes, as present in *Sinorhizobium meliloti* (Shatters *et al.*, 1993; De Bruijn *et al.*, 1989).

NH_3 + glutamate + ATP $\xrightarrow{GS}$ glutamine + ADP + P_i

Glutamine + 2-ketoglutarate + NADPH $\xrightarrow{GOGAT}$ 2 glutamate + $NADP^+$

NH_3 + 2-ketoglutarate + NADPH $\xrightarrow{GDH}$ glutamate + $NADP^+$

NH_3 + Pyruvate + NADPH $\xrightarrow{ADH}$ alanine + $NADP^+$

Figure 1.15 Enzyme catalysed reactions involved in the assimilation of ammonia

The current knowledge regarding ammonia assimilation in methanotrophs is based chiefly on the work of Murrell and Dalton (1983a,b) and is the source of the information below unless otherwise stated. These authors showed that *Methylomonas* S1, *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b possessed the GS/GOGAT pathway for assimilation of ammonia. In all three organisms, this was the sole pathway for ammonia assimilation at low ammonia concentrations (i.e. during growth on nitrate or nitrogen). At high ammonia concentrations (above 1 mM) the GS/GOGAT activity of both *Methylomonas* sp. S1 and *M. capsulatus* Bath decreased dramatically and was followed by the appearance of ADH and GDH activity for *Methylomonas* sp. S1, and ADH only for *M. capsulatus* Bath. However, ammonia assimilation in *Methylosinus trichosporium* OB3b appeared to proceed solely via the GS/GOGAT pathway, since the activity of this enzyme stayed relatively constant under all ammonia concentrations tested, and neither GDH or ADH activity was ever detected.

Only one ammonia assimilation gene has been cloned from a methanotroph and this is the *glnA* gene, encoding GS, from *Methylococus capsulatus* Bath (Cardy and Murrell, 1990). It possessed five highly conserved regions of homology found in both prokaryotic and eukaryotic GS enzymes. It was also possible to complement *glnA* mutants of *E. coli* with the *glnA* gene from *Methylococcus capsulatus* Bath.

1.9 Molecular biology and transcriptional control of nitrogen metabolism

Bacteria can utilise a wide variety of nitrogen sources, ranging from inorganic sources such as N_2, nitrate, ammonia and urea to complex organic sources such as histidine (Merrick, 1993). One organism often possesses several of the pathways for the assimilation of nitrogen and thus requires a tight regulation of the various pathways for nitrogen assimilation. This system is often called the nitrogen regulation system (*ntr*) (Merrick & Edwards, 1995). One protein, σ^N (formerly NtrA), is central to the regulation of several of these pathways. Section 1.7 describes the structure, proposed mechanism of transcriptional activation and describes the specific promoter sequences with which it interacts. σ^N was originally isolated as a gene required for the expression of glutamine synthetase in *Klebsiella pneumoniae* (Garcia *et al.*, 1977), and has subsequently found to be involved in regulation of several facets of the *ntr* system (Merrick, 1993).

The first facet of nitrogen metabolism with which σ^N is involved is in the fixation of nitrogen itself. As mentioned in section 1.7.1 the nitrogenase enzyme is encoded by a multigene operon including the *nifHDK* genes which encode for the nitrogenase enzyme subunits. Although σ^N was originally isolated as a gene required for glutamine synthetase expression, it is more often associated with the nitrogen fixation genes, *nif*. In many but not all nitrogen fixing bacteria *nif* gene transcription is dependent upon σ^N and NifA, an EBP (Merrick and Edwards, 1995). The classic model of *nif* gene regulation is illustrated by *Klebsiella pneumoniae* and *Azotobacter vinelandii*, where *nifA* is co-transcribed with the *nifL* gene (Merrick and Edwards, 1995; Halbleib and Ludden, 2000). The NifL protein is a flavoprotein containing an FAD group through which is it believed to sense the redox state of the cell (Dixon, 1998). Thus, in its oxidised form it is thought to inhibit NifA by direct protein-protein interaction (Merrick and Edwards, 1995; Dixon, 1998; Halbleib and Ludden, 2000). In contrast, NifA from *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* are not regulated by NifL. They contain four conserved cysteine residues in their C-terminal domains which are believed to bind a metal ion and directly sense the redox-state of the cell (Dixon, 1998; Morrett and Segovia, 1993). The situation in *Rhodobacter capsulatus* differs again, in that two copies of the *nifA* gene exist which respond differently to nitrogen levels (Halbleib and Ludden, 2000). It is clear that the

particulars of transcriptional regulation of nitrogen fixation differ across bacteria, but that σ^N plays a critical role in this process.

As discussed in section 1.7.2, assimilation of ammonia is an important process for the assimilation of nitrogen into cell biomass and the GS/GOGAT pathway is very important to many organisms. Transcriptional regulation of GS enzymes is intimately linked with σ^N. In enteric bacteria such as *Klebsiella pneumoniae* and *Escherichia coli* it is transcribed by the action of σ^N and the NtrC (an EBP) on promoters 5' of *glnA* (described in section 5.6.2). In these organisms *glnA* is organised in an operon with *ntrBC,* in the order *glnA-ntrBC,* and their transcription is linked (Merrick and Edwards, 1995). However, this is not the case in many non-enteric bacteria. Here *glnA* is often found in a *glnBA* operon e.g. *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* (Chiurazzi and Iaccarino, 1990), where its regulation is σ^N-independent, often constitutive and differs markedly between organisms (see section 5.6.2). As mentioned in section 1.7.2 the *glnA* gene from *Methylococcus capsulatus* Bath has been cloned and sequenced (Cardy and Murrell, 1990). The region 5' of *glnA* from *M. capsulatus* Bath contains both a putative σ^N-consensus promoter and a putative NtrC binding site, indicating that it may be regulated in a similar manner to the enteric bacteria. However, a gene encoding a homologue of NtrC (identified by hybridisation) is located 8.5 kb downstream of *glnA*, revealing that this *glnA* does not appear to lie in either a *glnBA* or *glnA-ntrBC* operon (Murell, 1992). It is known that the GS activity in *M. capsulatus* Bath is lost when cells are switched to high ammonia levels, and it is thus possible that under these conditions transcription from the σ^N promoter 5' of *glnA* in *M. capsulatus* Bath does not occur.

Many bacteria, including methanotrophs are able to utilise nitrate as sole nitrogen source. The genetics of nitrate assimilation has only been studied in very few cases. Transcription of the nitrate reductase genes (*nas*) is controlled by two mechanisms (Lin & Stewart, 1998). The first occurs under nitrogen limitation where transcription is activated by phosphorylated NtrC and σ^N (Lin and Stewart, 1998: Moreno-Vivian, 1999). The second involves induction of *nas* transcription by nitrate or nitrite (see section 5.6.2). Thus, like GS expression there are no hard and fast rules for transcriptional control of nitrate reduction genes. Thus making it difficult to predict the phenotype of an *rpoN*⁻ mutant of *Methylosinus trichosporium* OB3b or

Methylococus capsulatus Bath with respect to nitrogen metabolism, one of the aims of the work presented in Chapter 5.

1.10 Copper transport

It is widely accepted that copper is an essential trace element for the survival of all life forms from humans to bacteria (Mercer, 2001). Its ability to exist in two ionic forms [Cu(I)] or [Cu(II)] means it is present in enzymes essential to many organisms, including superoxide dismutase and cytochrome oxidases (reviewed by Peña *et al.*, 1999; Mercer, 2001). However, its ability to exist in distinct redox-states also means that it causes the production of highly reactive oxygen species, including hydroxyl radicals (Peña *et al.*, 1999), which can lead to cellular damage including lipid peroxidation, direct oxidation of proteins and cleavage of nucleic acid molecules. Since copper is both essential and potentially toxic to cells, its levels must be tightly regulated in order to prevent toxic accumulation or deficiency. This importance is illustrated by the defects in the Wilson and Menkes copper transport proteins in humans (Mercer, 2001). Wilson's disease is fatal unless treated and is caused by an accumulation of copper in the liver due to a defect in copper excretion. In contrast Menkes disease is caused by a defect in a copper uptake protein and so is characterised by symptoms caused by a lack of cuproenzymes. Both diseases are characterised by severe neurological conditions (Mercer, 2001).

As discussed above, specialised copper homeostasis mechanisms exist in all organisms. However, I will focus here on bacterial copper transport systems. These systems are characterised by possessing tightly regulated mechanisms for the uptake and efflux of copper (Peña *et al.*, 1999). The characterisation of such systems has revealed that a family of transmembrane proteins known as P-type ATPases facilitates copper transport across biological membranes in many organisms (Rensing *et al.*, 1999; Peña *et al.*, 1999; Solioz and Vulpe, 1996). The term 'P-type' refers to the formation of a phosphoenzyme intermediate in the reaction cycle (Rensing *et al.*, 1999; Solioz and Vulpe, 1996). Members of the 'P-type' ATPases are responsible for the transport of ions such as zinc, lead and cadmium in addition to copper (Rensing *et al.*, 1999). However, the copper-transporting 'P-type' ATPases form a subset of these proteins named the CPx-type ATPases, due to their possession of several distinguishing features (summarised in Figure 1.16) (Solioz and Vulpe, 1996).

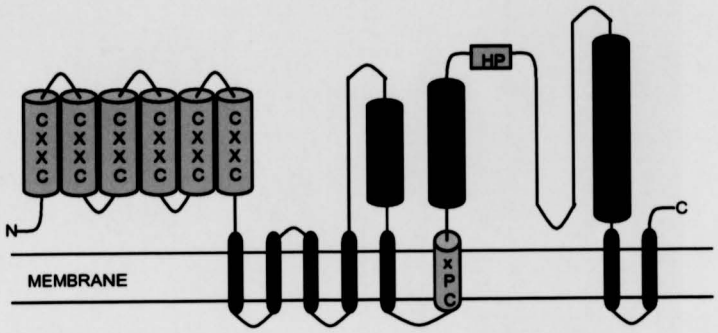


Figure 1.16 Schematic representation of CPx-type ATPAse. Features only present in CPx-type ATPases are highlighted in orange: 1. CXXC copper-binding motifs are present in 1-6 copies in CPx-type ATPases, 2. The characteristic CPx motif is believed to be involved in copper transport, 3. The highlighted HP motif is often mutated in Wilsons Disease patients.
The TGES phosphatase domain, DKTGT aspartyl kinase domain and GDGxNDxP ATP-binding domain are present in all P-type ATPases. (Adapted from Solioz and Vulpe, 1996).

The best characterised example of a CPx-ATPase copper transport system is the *copYZAB* operon from *Enterococcus hirae*. The CopA and CopB proteins are CPx-ATPases that are necessary for copper uptake and efflux from the cell, respectively (Solioz and Vulpe, 1996). Experiments by Solioz and Odermatt (1995) revealed that CopB was capable of causing cuprous (Cu[I]) copper and silver (Ag[I]) accumulation in native *E. hirae* 'inside-out' membrane vesicles, showing its role in efflux from the cell. Mutation of the CopA protein results in a strain that is impaired in growth under copper starvation conditions, indicating its role is in copper influx (Wunderli-Ye & Solioz, 2001; Odermatt *et al.*, 1993). A homologue of *E. hirae* CopB (named CopA) has now been characterised from *Escherichia coli* and has also shown to be responsible for the efflux of copper [I] ions from the cytoplasm (Rensing *et al.*, 2000; Petersen *et al.*, 2000).

In addition to CPx-type ATPases, several other, less well-characterised copper transport systems exist in bacteria. Recent work has revealed the presence of the *cusCFBA* operon, which is believed to encode a copper antiporter involved in copper ion efflux from the periplasm of *E. coli* cells (Grass and Rensing, 2001; Munson *et al.*, 2000). The *cus* determinant is homologous to the plasmid borne *pco* determinant which confers copper-resistance on *E. coli* strains isolated from the guts of pigs that had been fed a diet supplemented with copper sulphate (Brown *et al.*, 1995; Rouch

and Brown, 1997) and the *cop* operon from a strain of *Pseudomonas syringae* isolated from tomatoes sprayed with copper sulphate-containing herbicide (Mellano and Cooksey, 1988). A further gene, *cueO*, has also been identified in *E.coli* which is believed to encode a periplasmic multicopper oxidase, the mutation of which results in increased sensitivity to copper ions (Grass and Rensing, 2001). However, relatively little is known regarding copper uptake into *E. coli*, with the only candidates at present being the chromosomally encoded *cut* genes. These seven genes were identified by genetic studies, which revealed that their mutation conferred a range of copper sensitivity and accumulation phenotypes (Reviewed by Brown *et al.*, 1994)

The substrate for the CPx-type ATPases is probably Cu[I], although this is hard to prove definitively. Cells are usually presented with Cu[II] since Cu[I] is rapidly oxidised to Cu [II] in air (Greenwood & Earnshaw, 1984). However, the cytosol of cells is a highly reducing environment indicating the intracellular form of copper is likely to be Cu [I]. This is supported by the findings from copper transport assays using CopB from *E.hirae* which transports both Cu [I] and silver (Ag [I]) (Solioz and Vulpe, 1996; Rensing *et al.*, 1999). Several copper-responsive transcriptional activators from yeast are also responsive to Cu [I] and contain the characteristic Cu[I]-binding motif CXXC supporting the presence of Cu[I] in the intracellular environment (Koch *et al.*, 1997). If this is the case, then copper may be reduced from Cu[II] to Cu[I] during entry into the cell. Indeed, copper reducing enzymes have been identified in *Saccharomyces cerevisiae* (Georgatsou *et al.*, 1997; Hassett and Kosman, 1995), *Chlamyomonas reinhardtii* (Hill *et al.*, 1996), and *E.coli* (Rapisarda *et al.*,1999) among others (see section 7.6).

Of the bacterial copper transport systems listed above, all are subject to some form of copper-responsive gene regulation, ensuring expression of efflux proteins in the presence of excess copper and influx systems when the intracellular copper pool needs to be replenished. The currently known mechanisms of copper-mediated transcriptional regulation in bacteria are discussed in section 3.5.

1.11 Copper transport in methanotrophs

Although copper is fundamentally important to all bacteria, it is especially important to methanotrophs. Most methanotrophs express the pMMO enzyme, an enzyme which is believed to contain copper in its active site (Takeguchi *et al.*, 1999a,b; Zahn and Dispirito, 1996). In addition, the expression of both the sMMO and pMMO is controlled by the levels of copper ions in the growth medium (Stanley *et al.*, 1983; Nielsen *et al.*, 1996, 1997). However, relatively little is known regarding copper transport in methanotrophic bacteria.

The accumulation of copper in *Methylomicrobium album* BG8 has been studied and shows that copper uptake into these cells occurs at a faster rate than would be predicted by passive means, suggesting a dedicated copper uptake mechanism may function in methanotrophs (Berson and Lidstrom, 1996). Further evidence for a dedicated copper uptake system was provided by the discovery of small copper-binding compounds (CBCs) that are excreted into the growth medium of *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath (Tellez *et al.*, 1998; Zahn and Dispirito, 1996; Dispirito *et al.*, 1998). Two CBCs have been identified (see section 1.3.1). Their possible role in copper transport was highlighted by several pieces of evidence. Firstly, their maximum level of expression occurs during the switch from pMMO to sMMO expression, where the cells are copper stressed. Secondly, both CBCs bind copper (2-3 atoms per CBC), although the exact architecture of the copper co-ordinating centre is unknown. Thirdly, the sMMO-constitutive mutants PP319 and PP359 of *Methylosinus trichosporium* OB3b (Fitch *et al.*, 1993) produce high levels of CBCs constitutively and are unable to sequester CBC-bound copper (Dispirito *et al.*, 1998). The CBCs are also found in high levels in the membranes of wild-type *M. trichosporium* OB3b, suggesting that the CBCs are part of a pMMO specific copper-acquisition system (Dispirito *et al.*, 1998). This theory is also supported by the finding that mutants PP319 and PP359 do not contain CBCs in their membrane fractions, are unable to utilise CBCs and probably do not express pMMO. However, the fact that these mutants still accumulate 15-20% of copper levels of the wild-type and still contain functional copper-containing terminal oxidases, indicate that CBC-specific copper acquisition is not the sole means of copper acquisition in this organism (Dispirito *et al.*, 1998). If the CBCs represent part of a pMMO-specific copper acquisition system, then they may play an important role

in the environment where it is believed that pMMO is the chief form of methane monooxygenase that is expressed (Lidstrom and Semrau, 1995). Indeed, studies assessing the bioavailability of copper to *Methylosinus trichosporium* OB3b in different soil matrices suggest the CBCs may play a role in acquisition of soil-adsorbed copper (Morton *et al,* 2000).

The only gene to be implicated in copper transport is the *corA* gene from *Methylomicrobium album* BG8 (Berson and Lidstrom, 1997). This gene was identified by N-terminal sequencing of a copper-repressible polypeptide from membrane fractions from cells grown in medium containing high (40μM) and low (10 μM) copper concentrations. An analysis of its derived amino acid sequence showed it to have low identity (17.5% over 126 aa) with rabbit and human calcium channel proteins. A mutant of *corA* grew very poorly in NMS medium containing 0 μM, 10 μM and 50 μM copper indicating that it may play a role in copper transport in *M. album* BG8. However, its role in the transport of other metal ions cannot be ruled out since it has similarity to a calcium channel. A recent study revealed an outer membrane-glycoprotein, MopE, from *Methylococcus capsulatus* Bath, the C-terminal of which is cleaved and released into the growth medium (Fjellibirkeland *et al.,* 2001). The C-terminal part of this protein has significant homology to the derived amino acid sequence of *corA* (Fjellibirkeland *et al.,* 2001), and has been shown to bind 2 copper ions, indicating that it may be involved in copper transport either in it's cleaved or membrane-bound state (Ladstein, 1999). However, the physiological role of these proteins has yet to be established.

1.12 Aims of this thesis

The overall aim of this thesis was to increase understanding, at the molecular level, of the "copper-switch" mechanism. Most of the experiments presented in this thesis relate to *Methylosinus trichosporium* OB3b, since at the beginning of this project it was the most genetically amenable of the methanotrophs. The specific aims addressed are listed below:

1. Identification and characterisation of putative transcriptional start sites from the *pmo* gene clusters from *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M.
2. To investigate transcription of *pmo* and *mmo* genes in methane and methanol grown cells.
3. To establish the role of *rpoN* in regulation of the *mmo* operon.
4. To identify additional transcriptional regulation proteins involved in the "copper-switch"
5. To increase knowledge of copper transport systems in *Methylosinus trichosporium* OB3b.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

Plasmid manipulations and PCR products were transformed into *E. coli* strain TOP10 (Table 2.1). In those cases using streptomycin as selection marker, *E. coli* strain XL1-Blue was employed. Plasmids containing the RP4-*mob* conjugative transfer determinant were transferred from strain S17-1 to methanotrophs in direct bi-parental matings. Conjugative plasmids containing a streptomycin resistance cassette were transferred to methanotrophs using from strain JM109 in a tri-parental mating with the strain HB101 which contained the helper-plasmid pRK2013.

Table 2.1 ***Escherichia coli*** **strains**

Strain	Genotype	Reference
TOP10	F⁻, *mcrA, endA1, recA1*,ϕ80*lacZ*ΔM15, Δ*lacZX74*, *deoR, araD139, galK, rpsL* (Str^R), *nupG*, Δ(*mrr-hsdRMS-mcrBC*)	TOPO TA Cloning™ Kit Invitrogen Corporation
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB*⁺ *lacI*^{q}*ZΔM15 Tn10*(Tet^R)]	Stratagene, manual no. 200130
S17-1	*thi, pro, hsdR, hsdM, recA*, RP4 2-Tc::Mu-Km::Tn7	Simon *et al.*, (1983)
HB101	*recA thi leu pro hsdR* Sm^R	Boyer & Roulland-Dussoix (1969)
JM109	*endA1 hsdR17* (r_k^+ m_k^+) *supE44 thi recA1 gyr A96 rel A1* λ⁻ *Δ(lac-proAB)*(F'*traΔ36 proAB*⁺ *lacI*^{q} *lacZΔM15)*	Yannisch-Perron *et al.*, (1985)

Table 2.2 Methanotrophs and other strains

Strain	Source/ Reference
Methylosinus trichosporium OB3b	Warwick Culture Collection
Methylosinus trichosporium OB3b str. Gm1	This Study
Methylosinus trichosporium OB3b str. Gm2	This Study
Methylosinus trichosporium OB3b str. JS1	This Study (produced by Julie Scanlan)
Methylococcus capsulatus Bath	Warwick Culture Collection
Enterococcus hirae	Marc Solioz, Univerity of Berne
Sinorhizobium meliloti 1021	Philip Poole, University of Reading

Table 2.3 Plasmids

Plasmid	Description	Phenotype	Reference
p34S-Km	Km^R cassette with MCS	Ap^R, Km^R	Dennis & Zylstra (1998)
p34S-Gm	Gm^R cassette with MCS	Ap^R, Gm^R	Dennis & Zylstra (1998)
pTnModOKm	Tn5 plasposon suicide delivery plasmid	Km^R, Ap^R	Dennis & Zylstra (1998)
pUTMiniTn5Km	MiniTn5- suicide delivery plasmid	Km^R, Ap^R	DeLorenzo *et al.*, (1990)
pUTMiniTn5Sm/Sp	MiniTn5- suicide delivery plasmid	Sm^R/Sp^R, Ap^R	DeLorenzo *et al.*, (1990)
pRK2013	Helper plasmid for conjugative transfer used in tri-parental matings	Km^R, RK2-mob^+ (equivalent to RP4)	Figurski & Hellinski (1979)
pCR®2.1-TOPO®	General purpose PCR product cloning vector	Ap^R, Km^R	TOPO TA™ Cloning Kit, Invitrogen Corporation
pUC18	General purpose cloning vector	Ap^R, lacZ	New England Biolabs
pBR329*mob*	Mobilisable cloning vector	Ap^R,Cm^R, Tc^S, RP4-*mob*	Murrell, unpublished
pK18*mob*	Mobilisable cloning vector	Km^R, RP4-*mob*	Schaefer *et al.*, (1994)
pK18*mob*SacB	pK18mob containing SacB (levansucrase) sucrose-suicide gene	Km^R, Su^S	Schaefer *et al.*, (1994)
pMM17	*Klebsiella pneumoniae rpoN* gene in pBR325	Cb^R	Merrick & Gibbins (1985)
pAT705	*Azotobacter vinelandii rpoN* gene in pTZ18	Cb^R	Toukdarian & Kennedy (1986)
pSMEL5			

Plasmid	Description	Phenotype	Reference
pCOPY	*copY*-specific PCR product from *E. hirae* in pCR®2.1-TOPO®	Ap^R, Km^R	This study
pCOPZ	*copZ*-specific PCR product from *E. hirae* in pCR®2.1-TOPO	Ap^R, Km^R	This study
pCOPA	*copA*-specific PCR product from *E. hirae* in pCR®2.1-TOPO	Ap^R, Km^R	This study
pCOPB	*copB*-specific PCR product from *E. hirae* in pCR®2.1-TOPO	Ap^R, Km^R	This study
pMOPE5	*mopE*-specific PCR product from *M. capsulatus* Bath in pCR®2.1-TOPO	Ap^R, Km^R	This study
pGPS355	Putative *pqq*-biosynthesis cluster	Ap^R	This study
PGPS264	pUC18 containing 3 kb *Sal*I-*rpoN* containing fragment	Ap^R	This study
pGPS519	pUC18 containing 3.7 kb *Sst*I-*rpoN* containing fragment	Ap^R	This study
pGPS519Gm	pGPS519 containing *Bam*H1 Gm^R fragment from p34S-Gm inserted into *rpoN Bgl*II site	Ap^R, Gm^R	This study
pGPS519Km	pGPS519 containing *Bam*H1 Km^R fragment from p34S-Km inserted into *rpoN Bgl*II site	Ap^R, Km^R	This study
pGPS103	pBR329*mob* containing *Nco*I-*Hin*dIII fragment from pGPS519	Ap^R, Cm^S, Tc^S	This study

Plasmid	Description	Phenotype	Reference
pGPS103Gm	pGPS103 containing *Bam*H1 Gm^R fragment from p34S-Gm inserted into *rpoN Bgl*II site	Ap^R, Gm^R, Cm^S, Tc^S	This study
pGPS103Km	pGPS103 containing *BamH1* Km^R fragment from p34S-Km inserted into *rpoN Bgl*II site	Ap^R, Km^R, Cm^S, Tc^S	This study
pGPS104Gm	pK18*mob*SacB containing *Hin*dIII fragment from pGPS519Gm	Km^R, Gm^R	This study
pGPS110	pCR®2.1-TOPO® containing *rpoN* PCR product from M. capsulatus Bath	Ap^R, Km^R	This study
pGPS111	pBR329*mob* containing *EcoR1- rpoN* fragment from pGPS110, containing *Sal*1-Sm^R-fragment from p34S-Sm in *rpoN* gene	Ap^R,Sm^R, Sp^R,Cm^S, Tc^S	This study
pGPS112	pGPS110 containing *Sal1*-Gm^R fragment from p34S-Gm in *rpoN*	Ap^R, Km^R, Gm^R	This study
pGPS113	pBR329*mob* containing *EcoR1* fragment from pGPS112	Gm^R, Ap^R, Cm^S, Tc^S	This study
pGPS114	pK18*mob*SacB containing *Eco*R1 fragment from pGPS113	Gm^R, Km^R	This study

Ap, Ampicillin; Km, Kanamycin; Sm, Streptomycin; Sp, Spectinomycin; Gm, Gentamycin, Tc, Tetracycline; Cm, Chloramphenicol; Cb, Carbenicillin; SacB, levansucrase gene; *mob*, RP4-mobilisation determinant.

2.2 Growth media

All growth media were prepared using distilled water and sterilised by autoclaving at 15 pounds psi (121°C) for 15 min on a liquid cycle.

2.2.1 *Escherichia coli*

E.coli was routinely cultured in Luria-Bertani (LB) liquid medium (Sambrook, *et al.,* 1989). Long-term storage was achieved by the addition of 350 µl of sterile 50% (v/v) glycerol to 650 µl of a fresh overnight culture of *E.coli.* This was then mixed, frozen in liquid nitrogen and stored at –70°C. Strains were revived from frozen glycerol stocks by the transfer of an aliquot of frozen cell suspension (20 µl) to fresh LB medium containing the appropriate antibiotics. For preparation of solid media, 1.5% (w/v) of Bacto Agar (Difco, Michigan, USA) was added prior to autoclaving.

2.2.2 Methanotrophs

Methanotrophs were routinely grown in Nitrate Mineral Salts medium (1xNMS) media unless otherwise stated.

NMS Salts solution (x10 stock):

Potassium Nitrate	(KNO_3)	10 g
Magnesium Sulphate	($MgSO_4.6H_2O$)	10.79 g
Calcium Chloride	($CaCl_2.2H_2O$)	2.65 g

Dissolved in above order in 700ml and diluted to 1 litre

Iron solution (x10,000 stock):

Ferric-EDTA	(Fe-EDTA)	3.8 g

Final volume 100ml

Molybdate solution (x1000 stock):

Sodium molybdate	($NaMO_3$)	0.26 g

Final volume 1 litre

Trace elements solution (x1000 stock):

Copper Sulphate	($CuSO_4.5H2O$)	1 g
Ferric Sulphate	($FeSO_4.7H2O$)	2.5 g
Zinc Sulphate	($ZnSO_4.7H2O$)	2 g
Orthoboric acid	(H_3BO_3)	0.075 g
Cobalt Chloride	($CoCl_2.6H_2O$)	0.25 g
EDTA disodium salt	(Na_2.EDTA)	1.25 g
Manganese Chloride	($MnCl_2.4H_2O$)	0.1 g
Nickel Chloride	($NiCl_2.6H_2O$)	0.05 g

The above was dissolved in the specified order in distilled water and made up to five litres. This solution was stored in the dark at 4°C.

Phosphate buffer (x100 stock):

Disodium orthophosphate	($Na_2HPO_4.12H_2O$)	71.6 g
Potassium orthophosphate	(KH_2PO_4)	26 g

The above was dissolved in the specified order in 800 ml of distilled water. The pH was adjusted to 6.8 and diluted to 1 litre.

Media preparation:

1xNMS contains 100ml NMS salts, 1ml molybdate solution, 1 ml trace elements, 0.1 ml Fe-EDTA solution per litre, 10 ml of sterile phosphate buffer per litre was added after autoclaving.

Low-copper media was prepared using trace elements lacking copper. Trace elements, NMS salts, molybdate solution, Fe-EDTA and phosphate buffer were all prepared in acid-washed glassware using Milli-Q water.

Ammonia mineral salts medium (AMS) was prepared using a salt solution containing 10 g ammonium chloride (NH_4Cl_2) in place of potassium nitrate.

Nitrogen-free medium contained a salt solution lacking any nitrogen source.

Glutamine was added as a filter-sterilised stock solution (2.5%) to a final concentration of 0.05% (Murrell, 1981).

Agar plates were prepared by the addition of 1.5% Bacto agar (w/v) prior to sterilisation. Low-copper plates were prepared using 2% Noble agar (From Difco).

2.2.3 Antibiotics

Table 2.3 Antibiotics All antibiotics were added to medium after autoclaving and cooling, as filter-sterilised solutions.

Antibiotic	Dissolved in	Final concentration (μg/ml)
Ampicillin	Water	50
Carbenicillin	Water	50
Chloramphenicol	Ethanol	20
Gentamycin	Water	1-5
Kanamycin	Water	10-50
Nalidixic Acid	Water	10
Spectinomycin	Water	40
Streptomycin	Water	25
Tetracycline	Ethanol	10-25

2.3 Growth and maintenance of bacterial cultures

2.3.1 *Escherichia coli*

E. coli strains were routinely grown in 10 ml batch cultures at 37°C with shaking at 200 rpm. Antibiotics were added as appropriate to ensure plasmid maintenance. *E. coli* was also grown in larger volumes (50-1000 ml) under identical conditions.

2.3.2 *Sinorhizobium meliloti* 1021

Sinorhizobium meliloti 1021 was cultured at 30°C in LB medium containing 200 μg/ml streptomycin in 250 ml conical flasks sealed with cotton wool. It typically reached stationary phase after 3 days.

2.3.3 *Enterococcus hirae*

Enterococcus hirae is a class II pathogen and was grown on plates in the laboratory of Professor Chris Dowson under Class II containment conditions. The medium prepared contained: 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1% (w/v) Na_2HPO_4 .$2H_2O$, 1% glucose (added after autoclaving), 1.5 % (w/v) Bacto-agar. Growth was at 37°C and large colonies were formed after 2 days.

2.3.4 Methanotrophs

Methanotroph batch cultures were grown in 250 ml Quickfit flasks containing 50 ml of medium. Flasks were sealed with rubber Suba-Seals (W.H.Freeman, Barnsley,UK) and gassed by removing 60 ml air and injecting 60 ml methane/carbon dioxide (95%/5% (v/v) respectively). *Methylosinus trichosporium* OB3b was incubated at 30°C with shaking at 200 rpm, these cultures typically required about 5 days to reach stationary phase when an OD_{540}~0.8 was attained. *Methylococcus capsulatus* Bath was grown in a similar manner at 45°C.

Cultures were grown on agar plates containing the appropriate nitrogen source in sealed jars gassed under an atmosphere of 50% methane/carbon dioxide (95%/5% (v/v) respectively) and 50% air at 30°C or 45°C as appropriate. Colonies of 2 mm diameter typically formed within 7 days.

Chemostat cultures of methanotrophs were grown at 30°C or 45°C where appropriate in a 2 or 4 litre fermenter vessels (L.H. Engineering) with an agitation speed of 500-700 rpm., and gas flow rates of 60ml min^{-1} CH_4 (2 litre vessel), 120ml min^{-1} CH_4 (4 litre vessel); 300ml min^{-1} air (2 litre vessel), 600ml min^{-1} air (4 litre vessel). NMS medium containing 0.1 mgl^{-1} or 1mgl^{-1} of added $CuSO_4$ was used for low and high-copper media as appropriate. A typical dilution rate was 0.03 h^{-1}. The pH was maintained at 6.8 by the automatic addition of sterile 0.5 M HCl or 0.5 M NaOH. Fresh late exponential phase (OD_{540}~0.6) cultures of methanotrophs were used as inocula (10% v/v).

2.3.5 Purity checks of methanotrophic cultures

Methanotroph cultures were routinely checked for purity by streaking onto nutrient agar (NA) plates (Sambrook *et al.,* 1989) and incubating aerobically at 30°C for two days. Methanotrophs have an obligate requirement for methane and so growth on NA plates indicated contamination. The cultures were also checked using routine light microscopy using a Kyoga-Unilinx III (Tokyo) phase-contrast microscope at 1,000X under oil-immersion.

2.4 General pupose buffers /solutions

Agarose gel-loading buffer (6x)

Bromophenol blue	0.0125 g
Ficoll (Type 400)	0.75 g
Distilled water	5 ml

TE Buffer (pH8.0)

Tris-HCl	10mM
Na_2EDTA	1mM

10xTBE Buffer (pH8.0)

Tris base	135 gl^{-1}
Boroc acid	55 gl^{-1}
Na_2EDTA	9.3 gl^{-1}

20x Standard Saline Citrate (SSC)

NaCl	173.3 gl^{-1}
Tri-sodium citrate	88.2 gl^{-1}

Dissolve in 800ml distilled water and adjust pH to 7.0 before making up to 1 litre.

2.5 DNA extraction

2.5.1 Plasmid extraction from *E.coli*

2.5.1.1 Small scale plasmid extraction (minipreps) by alkaline lysis

Small scale plasmid extractions were performed on 1.5 ml *E.coli* cultures grown for 16 h, according to the method of Saunders and Burke (1990).

2.5.1.2 Large scale (maxipreps) by alkaline lysis

Large scale plasmid DNA extractions were performed on 500 ml - 1 litre of stationary phase *E.coli* cultures, grown in LB with the appropriate antibiotics (Table 2.3), according to the alkaline lysis method of Sambrook *et al.*, (1989). The resultant nucleic acid pellet was dissolved in TE buffer. Caesium chloride (CsCl) was added to a concentration of 1 gl^{-1} (w/v). 0.8 ml ethidium bromide (10 $mgml^{-1}$) was added per 10 ml DNA/CsCl solution. The mixture was placed in a quick seal tube (Beckman, Buckinghamshire, UK) and the remaining volume of the tube filled with 1 gl^{-1} (w/v) CsCl dissolved in TE. The mixture was centrifuged at 196,000 x *g* for 16 h at room temperature in a Beckman Vti50 rotor. This allowed the separation of different types of DNA according to their buoyant densities. Bands were visualised using a hand-held UV lamp and the lower band, containing the closed circular plasmid DNA was removed from the gradient with a syringe fitted with a large gauge hypodermic needle.

Ethidium bromide was removed using TE-saturated butanol until the mixture became clear. The DNA was then precipitated (section 2.6.2) and resuspended in distilled water.

2.5.2 Extraction of DNA from methanotroph cultures

DNA was extracted using the method for methanotrophs described by Oakley and Murrell (1988). Further purification of the DNA was achieved by dialysing the DNA solution against 2 litres TE buffer for 24 h (3 changes of buffer solution) at 4 °C.

2.5.3 Extraction of DNA from *Enterococcus hirae*

DNA was extracted from *Enterococcus hirae* cells which had been removed from agar plates using a sterile disposable spreader and resuspended in 500 µl TE by the following method:

1. 80 µl of lyzozyme solution (lyzozyme 10mgml^{-1} in TE) was added to 500 µl of cell suspension and incubated for 10 min at 25 °C.
2. 15 µl 20% (w/v) SDS, 6 µl proteinase K (10 mgml^{-1}) was then added before incubation for 1 h at 37 °C.
3. 100 µl 5 M NaCl and 80 µl 10 % (v/v) Cetyltrimethyl ammonium bromide (CTAB)/ 0.7 M NaCl was then added before incubation for 10 min at 65 °C.
4. DNA was extracted with 800 µl chloroform, by centrifugation at 10,000 x *g* followed by removal of the aqueous phase.
5. 800 µl phenol:chloroform (1:1) was then added, mixed and extracted as in step 4.
6. DNA was then precipitated with 450 µl isopropanol and mixed by inversion.
7. DNA was then collected by centrifugation at 10,000 x *g* and washed with 70 % (v/v) ethanol, air dried for 3 min and resuspended in 50 µl TE.

2.6 Nucleic acid techniques

2.6.1 Phenol/chloroform DNA extraction

Phenol, chloroform and isoamyl alcohol were mixed in the ration 25:24:1 (v/v) respectively and stored in the dark at 4°C. In order to extract protein from DNA solutions an equal volume of phenol/chloroform/isoamyl alcohol solution was added to the DNA and mixed by vortexing for 30 sec. This was then centrifuged at 20,800 x *g* for 2 min and the (upper) aqueous phase retained. Phenol was then removed by mixing with an equal volume of chloroform/isoamyl alcohol (24:1), followed by centrifugation. The aqueous phase was then removed and the DNA precipitated as described in section 2.6.2.

2.6.2 Precipitation of DNA

DNA was routinely precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% (v/v) ethanol to the DNA solution. This was then incubated for 30 min to 2 h at -20°C. The DNA was collected by centrifugation at 20,800 x *g* at 4°C for 20 min. The resulting pellet was washed in 70% ethanol (v/v) and air-dried before resuspension in sterile distilled water.

2.6.3 Quantification of DNA

Routine quantification of DNA was achieved by estimation of DNA solutions by running on a TBE-agarose gel (Section 2.6.7) and comparison of DNA samples with known quantities of DNA standards. DNA standards used were either 1kb ladder (GIBCO BRL) or *Hin*dIII digested lambda DNA (GIBCO BRL).

2.6.4 Restriction endonuclease digestion of DNA

Restriction endonucleases and buffer solutions were supplied by GIBCO BRL and New England Biolabs. Restriction digestion of DNA were performed according to the manufacturers instructions.

2.6.5 Dephosphorylation of DNA

High activity calf intestinal alkaline phosphatase (CIAP) was obtained from Boehringer Mannheim (East Sussex, UK) and used according to the manufacturers instructions.

2.6.6 Ligation of DNA

DNA ligations were performed using T4 DNA Ligase from GIBCO BRL using the following reaction mixture:

Vector DNA	~50 ng
Insert DNA	~100 – 500 ng
10 mM ATP	2µl
5x Ligase buffer	4µl
T4 DNA Ligase	1µl (1Unit)
Distilled water	Up to total volume 20 µl

Several vector:insert ratios were typically used. Ligation reaction mixtures were incubated overnight at 16°C and 10 µl was used in subsequent transformations (section 2.8.1).

2.6.7 Agarose gel electrophoresis

Agarose gels were prepared and run in 1 x TBE buffer. Small gels were run on Flowgen minigel systems (Flowgen Instruments Ltd., Sittingbourne, UK) and larger gels were run on BRL model H4 horizontal gel systems (Bethesda Research laboratories, Cambridge, UK). Ethidium bromide was added to a final concentration of 0.5 $\mu g ml^{-1}$ directly to the gel before casting. DNA was visualized on a UV transilluminator and gels were photographed using an instant camera (CU5 Land camera) loaded with Polaroid 665 black and white film. 1 kb ladder (Gibco BRL) and *Hin*dIII digested Lambda 'phage DNA (Gibco BRL) were used as size standards.

2.6.8 Extraction of DNA from Agarose gels

DNA fragments were recovered from TBE-agarose gels by excision of the appropriate region of the gel and extracted using the GENECLEAN®II Kit (Bio101, California, USA) according to manufacturers instructions.

2.7 Construction of partial libraries of DNA from *Methylosinus trichosporium* OB3b

Partial genomic libraries were constructed by cloning DNA from *Methylosinus trichosporium* OB3b into the multicopy vectors pUC18 or pUC19. The vector was prepared by digestion with the appropriate restriction endonuclease followed by treatment with CIAP to remove 5'-phosphate groups in order to minimise self-ligation of the vector. DNA from *Methylosinus trichosporium* OB3b was completely digested with the same (or compatible) enzyme followed by fractionation through an agarose gel. The desired size fraction was then excised from the gel and the DNA extracted as detailed in section 2.6.8. The recovered fragments were then ligated (Section 2.6.6) into pUC18 and transformed into *E.coli* XL1-Blue or TOP10 (section 2.8).

In the case of pUC18, the successful ligation of insert DNA into the vector was indicated by the insertional activation of the *lacZ* gene within which the multiple

cloning site resides. This was visualized by the inclusion of 40 μgml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 10 mM IPTG (isopropyl-1-thio-β-Dgalactopyranoside) for *E.coli* XL1-Blue in the agar plates. A clone with an intact *lacZ* gene will convert X-Gal into a blue product, whereas an insertionally inactivated *lacZ* gene will remain 'white'. In this way, 'white' clones with inserts were selected.

2.8 Transformation of *E.coli*

2.8.1Transformation by chemical competence

Competent cells were prepared by treatment with cold-calcium chloride. A fresh overnight culture of *E.coli* was inoculated into 50 ml fresh LB and allowed to grow to an OD_{600} of 0.5 (typically 2 h). This culture was then placed on ice for 10 min in a chilled JA20-centrifuge tube (Beckman Ltd.) before centrifugation at 4°C in a Beckman JA20 rotor for 10 min at 4,000 rpm. The cell pellet was then gently resuspended in 10 ml of ice-cold $CaCl_2$ and left on ice for 10 min before centrifugation. This pellet was then resuspended in 1 ml ice-cold $CaCl_2$ and 200 µl aliquots were placed in chilled 1.5 ml Eppendorf tubes. Cells were used for transformations immediately or frozen in liquid-nitrogen and stored at –70°C.

Transformation was achieved by the addition of 10-100 ng of plasmid DNA or 10 µl of a ligation mixture to the cell suspension, incubation on ice for 30 min to 2 h, before heat shock at 42°C for 1 minute. The transformation mixture was placed on ice to recover for 2 min prior to the addition of 800 µl SOC (2% (w/v) bacto-tryptone; 0.5% (w/v) yeast extract; 10mM NaCl; 2.5mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$; 20mM glucose) and incubation at 37°C with shaking (200rpm) for 1 h (expression step). Cells were then plated on LB agar plates containing the appropriate plasmid selection. This method typically resulted in transformation frequencies of $1x10^6$ to $1x10^7$ per µg DNA.

E.coli strains typically used were TOP10 and XL-1 Blue (Table 2.1) depending on requirements.

2.8.2 Electrotransformation of *E.coli*

A Bio-Rad GenePulser™ and Bio-Rad PulseController™ were used for electroporation, according to the manufacturers instructions. A 10 ml overnight culture of *E. coli* was washed three times in ice-cold sterile distilled water (Milli-Q) and resuspended in 200µl ice-cold sterile distilled water. Approximately 10-100ng of plasmid DNA was added to 40 µl aliquots of cell suspension followed by electrotransformation using 0.2 cm electroporation cuvettes. The settings used were 12.5 $kVcm^{-1}$, 25 mF, 200Ω. The cells were placed on ice for 1 min to recover and 1ml LB was then added before incubation at 37°C for 1 h. Cells were then plated out as described previously. This method typically resulted in transformation frequencies of 1 x 10^6 to 1x 10^8 transformants per µg DNA and was the method of choice for transformation of *E. coli* strain S17-1.

2.9 Conjugal transfer of plasmids to methanotrophs by filter mating

The procedure for conjugating plasmids from *E.coli* into methanotrophs was based upon the method developed by Martin and Murrell (1994). The RP4-*mob*-containing plasmid to be conjugated was first transferred into *E. coli* S17-1, which contains the RP4 transfer functions in its chromosome, thus promoting high frequency mobilization of plasmids containing the RP4-*mob* sequence. For marker exchange mutagenesis plasmids, a narrow host-range vector backbone, which will not replicate in the methanotroph, was used, e.g. pBR329*mob*, pK18*mob*; thus creating a suicide vector.

A 10 ml overnight *E.coli* culture containing the donor plasmid was collected on a sterile 47 mm nitro-cellulose filter (0.2 µm pore size; Millipore). The *E. coli* donor strain was then washed on the filter with 50 ml sterile NMS (or appropriate methanotroph media). A 50 ml culture of the methanotroph was grown to an OD~0.3 and collected on the same filter as the *E.coli* donor strain, and again washed with 50 ml media. The filter was then placed (cells up) on an NMS (or other) agar plate containing 0.02 % (w/v) proteose peptone and incubated for 24 h at the appropriate temperature in the presence of methane.

After 24 h the cells on the filter were resuspended in 10 ml sterile medium by vortexing. These cells were then concentrated by centrifugation in a Mistral 100

swing rotor (MSE, Loughborough,UK) at 7,000 x *g* for 10 min. The pellet was resuspended in 1 ml sterile medium and 100 µl aliquots were spread onto selective agar plates. The plates were then incubated until colonies formed, typically 10-14 days. The background *E. coli* present was removed by streaking the resultant colonies onto nalidixic acid ($10\mu gml^{-1}$) containing plates. The methanotrophs used here are Nal^R, whereas the *E. coli* are Nal^S.

2.10 Southern transfer of DNA

The Southern blotting method outlined in Sambrook *et al.* (1989) was used to transfer DNA onto nylon Hybond-N membranes (Amersham, Little Chalfont, UK). DNA was fixed to the membrane with an Ultraviolet (UV) Stratalinker (Stratagene, Cambridge, UK).

2.11 Colony blots

Colonies were picked onto a grid-marked Hybond-N membrane placed on the surface of agar plates and grown overnight in the case of *E.coli* and 4 days for *Methylosinus trichosporium* OB3b. After growth, the cells were lysed by the following procedure:

3MM Whatman paper cut 5mm larger than the filter was soaked in the solutions listed below and the filter was then placed on top of these Whatman papers. Denaturing and neutralising solution were made up as described in Sambrook *et al.* (1989).

10 % SDS	10 min
Denaturing solution	10 min
Neutralising solution	5 min
2 x SSC	5 min
Dry (room temperature)	30 min

DNA was fixed to the membrane with an Ultraviolet (UV) Stratalinker (Stratagene, Cambridge, UK).

2.12 Hybridisation of DNA

All hybridisations were carried out in a Hybaid oven at 65°C unless otherwise stated. Hybridisation was made by mixing 0.5 M Na_2HPO_4 and 0.5 M NaH_2PO_4 in roughly equal amounts to give a pH in the range of 6.6-7.2, then adding 7% (w/v) SDS and 5 mM EDTA. This solution must be heated to 50°C until clear, before use.

A pre-hybridisation step was performed where the membrane was wrapped in mesh and placed in a Hybaid tube. 20 ml of hybridisation was added before incubation at 65°C for 30 min. After 30 min, the hybridisation solution was replaced with fresh solution and the purified probe added. Hybridisations were typically carried out overnight at 65°C.

Stringency washes typically began at 2 x SSC room temperature, low stringency, and worked there way through increasing temperature and decreasing concentrations of SSC in increments to a maximum stringency of 0.1 x SSC, 80°C.

2.13 Radiolabelling of DNA by Random Priming

Labelling of DNA probes was performed by random priming according to Feinberg and Vogelstein (1984). 50 ng of PCR-generated was labelled with 50 μCi of [α-^{32}P]dGTP. Unincorporated label was removed using a MicroSpin™ Column (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK) according to the manufacturer's instructions. Probes were denatured by the addition of NaOH to a final concentration of 0.4 M immediately prior to addition to hybridisation solution.

2.14 Autoradiography

Fuji nif RX medical X-ray film was used for all autoradiographs. Radioactive membranes were exposed to this film in light-tight autoradiography cassettes with two intensifying screens. Cassettes were stored at –70°C with the length of exposure (typically between 1 and 72 h) being dependent upon the signal intensity. Autoradiographs were developed and fixed in accordance with the manufacturer's instructions.

2.15 Polymerase Chain Reaction (PCR)

PCR amplifications were performed in a total volume of 50 µl in 0.5 ml microfuge tubes using a Hybaid Touchdown™ Thermal Cycling System.

Typical reactions contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 250 mM of each nucleotide, 100 ng of each primer and 2.5 Units of *Taq* polymerase (Gibco BRL) which was added after a 5 min 'Hot-start' at 94°C. Template concentrations were optimised by testing several dilutions of template stock. Normal reaction conditions were:

'Hot Start'	94°C	5 min
	Add *Taq* polymerase	
Denaturing	94°C	1 minute
Annealing	Varied with primer	1 minute
Extension	72°C	1 minute

30 cycles

Final cycle with extension for 10 min.

Difficult PCR reactions were performed using a Touchdown protocol using a reaction mixture including 25 µl DMSO-Betaine 2 x stock (2 x Stock, 2.6 M Betaine, 2.6 % (v/v) DMSO). This typically began with a Hot-Start as shown above, followed by addition of *Taq* ploymerase. If the predicted annealing temperature was 54°C, then this was followed by 20 cycles with annealing temperatures decreasing (1 °C per cycle) from 73-53°C with denaturing and extension as described above. After this a further 15 cycles would be carried out at an annealing temperature of 54°C, followed by a final cycle with an extension step of 10 min.

2.16 DNA sequencing and analysis

2.16.1 DNA sequencing

DNA sequencing was performed by L. Ward (University of Warwick) by cycle sequencing with the Dye Terminator Kit (PE Applied Biosystems, Warrington, UK) and analysed using a model 373A automated sequencing system (PE Applied Biosystems).

2.16.2 Sequence analysis

DNA sequences and derived amino acid sequences were analysed using the DNAStar package. Similarity searches were performed using the gapped BLAST (Basic Local Alignment Search Tool) program (Altschul *et al.*, 1997) against public protein and gene databases (http://www.ncbi.nlm.nih.gov).

2.16.3 Sequence alignments and phylogenetic analysis

Deduced amino acid sequences were aligned using the CLUSTAL W program. Alignment positions where sequence ambiguity existed and where data were not available for all sequences were excluded from the analysis.

The phylogeny of the *rpoN* sequences was determined with programmes available within PHYLIP (Felsenstein, 1993). An evolutionary distance matrix prepared using the Dayhoff PAM parameter model (PROTDIST) was used for construction of phylogenetic trees with the FITCH programme. The significance of the branch points was assessed by bootstrap resampling of the dataset (SEQBOOT). Bootstrap values were obtained from the consensus (CONSENSE) of 100 trees generated by evolutionary distance (PROTDIST) analysis.

2.17 RNA extraction from methanotrophs

All solutions listed below were made using Diethylpyrocarbonate (DEPC) - treated water, using fresh tips, gloves and clean Gilson pipettes at all times.

DEPC-treated water was prepared by treating distilled water for 1 h at 37°C with 0.1 % (v/v) DEPC followed by autoclaving for 15 min at 15 psi on liquid cycle.

RNA was extracted from 50 ml of late-exponential phase (OD_{540} = 0.5) culture. Cells were harvested by centrifugation in sterile disposable 50 ml Sarstedt plastic tubes in a Mistral 100 swing rotor (MSE, Loughborough,UK) at 7,000 x *g* for 10 min before resuspension in 200 µl Solution 1 (0.3 M sucrose; 0.01M sodium acetate, pH 4.5). After resuspension, 200 µl Solution 2 (2% SDS; 0.01 M sodium acetate, pH 4.5) was added. This cell suspension was then transferred into Ribolyser Blue tubes (Hybaid) before the addition of 400 µl of RNAse free acidic phenol (saturated with 50 mM sodium acetate, pH 4.5). Next, cells were placed on ice before lysis by a 30 sec pulse at Speed 6 m/s in a Hybaid Ribolyser. After 2 min on ice the mixture was centrifuged at 10,000 x *g* and the aqueous layer transferred to a fresh

Eppendorf tube. 400 µl of acidic phenol were added and after mixing, the solution was placed at 65°C for 2 min before drop-freezing in a dry-ice ethanol bath. The solution was thawed on ice before spinning at full speed for 5 min. The aqueous phase was placed in a fresh Eppendorf and 400 µl of 1:1 phenol:chloroform added. After mixing this was centrifuged and the aqueous phase placed in a fresh tube. 40 µl of RNAse free 3 M sodium acetate (pH 4.5) and 900 µl of 96% (v/v) reagent grade ethanol (Sigma) was added before placing at –20°C for 20 min and centrifugation for 20 min. After precipitation the pellet was washed in 70% (v/v) ethanol before drying. Pellets were resuspended in 40 µl of DEPC treated sterile H_20. RNA quality was checked by running 2-5 µl of RNA solution on a 1.2% (w/v) TBE-agarose gel in RNA-loading buffer (1 ml formamide; xylene cyanol FF 1 mg; bromophenol blue 1 mg; 200 µl 0.5 M EDTA, pH 8.0). Removal of DNA from the samples was achieved using RQ1 DNAse from Promega as per manufacturers instructions. The presence of *pmo/mmo*-specific DNA fragments was tested by PCR using primers specific for these operons (Se Figure 4.1). RNA was quantified in Quartz cuvettes in a Beckman DU-70 at a wavelength of 260 nm.

2.18 Reverse Transcriptase-PCR

The reverse transcription step was essentially performed as per manufacturers instructions (Roche). Briefly, 1µg of DNA-free RNA was added to 50 pmol primer and water up to a final volume of 4.5µl. The mixture was then heated to 65°C for 15 min in a Hybaid Touchdown™ Thermal Cycling System to denature the RNA, followed by snap-cooling on ice for 2 min. To this mixture, 2 µl each dNTP (10 mM), 2 µl 100 mM Dithiothreitol (DTT), 4 µl 5 x Expand buffer, 0.5 µl water and 1 µl Expand Reverse Transcriptase (40 units/µl) were added before incubation at 42°C for 1 h. PCR was then performed on 1 µl of the RT-reaction mixes to determine the presence/absence of the target-gene specific RNA-derived cDNA in the samples. PCR reactions were performed as described in Section 2.15. The products of the PCR reaction were assessed on TBE-agarose gels (Section 2.6.2).

2.19 Primer extension

2.19.1 End labelling of primer

Primers were obtained from Gibco BRL. They were typically 18-mers designed in the reverse orientation 3' of the region to be mapped by primer extension. The labelling reaction was made up as follows:

Primer	1 µl (10 pmol)
T4 Kinase forward reaction Buffer (10x)	2 µl
γ-^{32P}-ATP (500 Ci/mmol)	2 µl
T4 Kinase	1 µl
dH_2O	14 µl

This mixture was incubated at 37°C for 1 hour, after which the reaction was halted by incubation at 70°C for 10 min.

2.19.2 Hybridisation and extension reaction

10 µg of RNA was dissolved in 2.5 µl of RNAse free sterile distiled water and mixed with 1 µl [γ-^{32P}]ATP-labelled primer and 1 µl of 4.5 x hybridisation buffer (4.5 x hybridisation buffer contains: 250 mM HEPES (pH 7.0), 500 mM KCl). This mixture was denatured by heating to 75°C for 1 min followed by gradual cooling to 30°C over a period of 1 h. To the denatured mixture, 3 µl extension mix (260 mM Tris HCl (pH 8.4), 20 mM $MgCl_2$, 20 mM Dithiothreitol, 0.2 mM each dNTP) and 1.6 Units of avian myoblastosis virus (AMV) reverse transcriptase (Promega) was added. The extension reaction was incubated at 45-50°C for 30 min. The extension products were precipitated on ice for 20 min with 1 µl 3 M sodium acetate (pH 4.5) and 20 µl 96% (v/v) ethanol, followed by spinning and washing with 70% (v/v) ethanol. After drying, the products were resuspended in 6 µl of RNA loading buffer (section 2.17). The products were heated to 75°C for 2 min immediately prior to running onto an 8% (w/v) polyacrylamide gel alongside a set of dideoxy sequencing products of the appropriate plasmid DNA template with the same primer. Sequencing reactions were performed as described in section 2.19.3.

2.19.3 Manual sequencing of plasmid DNA

Sequencing reactions were carried out according to the manufacturers instructions (Sequenase version 2.0, DNA Sequencing Kit; USB). The primers used corresponded with those used for primer extension reactions.

2.19.4 Gel preparation

Sequencing gel mix (8% w/v) for two gels contained:

Acrylamide (40% stock)	20 ml
Urea	8 g
10 x TBE (Fresh)	10 ml
Water	up to 100 ml
Warm to dissolve.	

Standard sequencing gel tanks 38 x 50 cm were prepared for use by thorough washing in hot water and 10 gl^{-1} KOH dissolved in methanol. Immediately before pouring, the plates were washed 3 times with distilled water and twice with 100 % ethanol. The top plate was then treated with Repelcote® (BDH, Poole, UK). 4mm spacers were inserted between the plates, which were sealed at the bottom and side edges with tape and clamped. Immediately prior to casting 500 µl 10% (w/v) ammonium persulphate and 65 µl *N, N, N', N'*-tetramethylethylenediamine (TEMED) were added. A sharks tooth comb was then inserted and the gel allowed to polymerise for 1 h. Gels were pre-run at 40 W constant power for 30 min. The wells were then washed out with 1 x TBE running buffer to remove excess urea and acrylamide, prior to loading. Samples were denatured by heating to 75°C for 2 min immediately before loading. Primer extension products (3 µl) were run alongside their corresponding sequencing reaction tracks (4 µl). The gels were run at 40 W constant power until the blue dye front reached the bottom (typically 3 h). After electrophoresis, the glass plates were carefully split and the gel transferred onto a piece of 3MM Whatman paper. This was then covered in cling film and dried using a gel drier (Bio-Rad) before exposure to Fuji nif RX medial X-ray film. Exposure times were typically 12-36 h. Autoradiographs were developed as described in section 2.13.

2.20 SDS-PAGE

2.20.1 Gel preparation

Protein-containing samples were analysed by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). A 4 % (w/v) stacking gel and 12 % (w/v) separating gel were typically used.

Stacking gel	
Water	6.1 ml
0.5 M Tris-HCl (pH 6.8)	2.5 ml
10 % (w/v) SDS	100 µl
30 % Acrylamide stock (29:1)	1.3 ml
10% (w/v) Ammonium persulphate (fresh)	50 µl
TEMED	10 µl
Total volume	10 ml

Separating gel	
Water	3.5 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml
10 % (w/v) SDS	100 µl
30 % Acrylamide stock (29:1)	4 ml
10% (w/v) Ammonium persulphate (fresh)	100 µl
TEMED	10 µl
Total volume	10 ml

Running buffer (5x) consists of:

Tris base	15 gl^{-1}
Glycine	72 gl^{-1}
SDS	5 gl^{-1}
Water	up to 1 litre

Electrophoresis was performed in an X-Cell II^{TM} MINI-Cell (NOVEX, San Diego, USA) at 80 mA constant current for 1 hour. Proteins were visualised using

Coomassie Brilliant Blue (0.1 % Coomassie Blue R-250 in fixative: 40 % methanol, 10 % Glacial acetic acid, 50% water (v/v)), and de-stained in 40% (v/v) methanol plus 10 % (v/v) glacial acetic acid.

SDS-PAGE gels were calibrated using Amersham Low Molecular Weight markers: α lactalbumin, 14 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; albumin, 67 kDa; phosphorylase b, 94 kDa. Gels were scanned using a BioRad GelDoc system. Markers and protein samples were mixed with at least an equal volume of SDS sample buffer (1 ml 0.5M Tris H-Cl (pH 6.8); 8 ml Glycerol; 1.6 ml 10 % SDS; 0.4 ml 2-β-mercaptoethanol; 0.2 ml 0.05 % bromophenol blue ; 4 ml water) and boiled for 5 min prior to loading.

2.20.2 Whole cell protein extract preparation

Cells were harvested by centrifugation from late-exponential phase cultures as described in section 2.16. The pellets were resuspended in 200 µl 20 mM Tris-HCl (pH7.3) and placed in chilled Ribolyser Blue tubes. The cells were then lysed by pulsing in a Hybaid Ribolyser for 10 seconds at speed 6 m/s, followed by 2 min on ice. This process was repeated 5 times. The cell lysate was then centrifuged at full speed (10,000 x *g*) and the supernatant collected in a fresh 1.5 ml Eppendorf tube.

2.20.3 Protein quantification

Total protein concentration was determined using Bio-Rad protein assay reagent according to the manufacturer's instructions. Bovine Serum Albumin (BSA) was use as a standard.

2.21 Western blotting

Following electrophoresis, the SDS-polyacryamide gel was soaked for 30 min in transfer buffer (10 x Stock: 30.3 gl^{-1} Tris base, 146 gl^{-1} glycine, 10 gl^{-1} SDS, water up to 1 litre; 1 x transfer buffer: 100 ml 10 x stock, 200 ml methanol, water up to 1 litre). Proteins were electro-blotted on to Hybond-C nitrocellulose membrane (Amersham) in a Novex X-Cell II blot module at 25 V constant voltage for 2 h.

The membrane was then soaked in 2 % (w/v) skimmed milk powder in 1 x TBST (20 x TBSTcontains: 1 M Tris HCl (pH 7.4), NaCl 180 g, 1 % (v/v) Tween 20, water to 1 litre) for 1 hour at room temperature or overnight at 4°C to block non-

specific protein-binding sites on the filter. After equilibration, the filter was incubated with the appropriate dilution of primary antiserum, diluted in TBST with 2% (w/v) skimmed milk powder, for 90 min at room temperature with agitation. For sMMO hydroxylase antibody, 400 µl in a volume of 20 ml was used. The filter was then washed in TBST 3 times for 10 min before incubation for 2 h with 50 µl secondary antibody in 20 ml TBST plus 2% (w/v) skimmed milk powder. The secondary antibody was a goat-anti rabbit IgG (Sigma) conjugated to Horse Radish peroxidase enzyme. The filter was rinsed with TBST twice for 10 min followed by two washes in TBS (TBST without the Tween 20). The antibody-peroxidase conjugate was visualised by incubation for 10 min with staining solution, Development was halted by repeated washing with distilled water.

Staining solution was made as follows:

Solution A	
NaCl	1.5 g
1 M Tris-HCl (pH 6.5)	1 ml
Water up to 50 ml	

Solution B	
Chloronaphthol (Sigma)	1 x 30 mg tablet
Methanol	10 ml
Water	made up to 50 ml after dissolving Chloronaphthol in methanol

Immediately prior to use 50 µl of hydrogen peroxide (100 volumes) was added to solution B which was then combined with solution A.

2.22 2D-SDS PAGE

2.22.1 Sample preparation and cell fractionation

This method was developed by Frøde Berven, Odd André Karlsen and Dr Anne Fjellbirkeland, University of Bergen, Norway (Fjellbirkeland *et al.*, 1997). Cells were harvested from chemostat cultures of *Methylosinus trichosporium* OB3b by centrifugation, resuspended in 20 mM Tris-HCl (pH 7.3), and drop frozen in liquid nitrogen. One gram of frozen cell pellet (wet-weight) was diluted with 5 ml of 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$ and 200 µl was removed: Whole Cells [Sample 1]. The cells were then lysed by three passages through a French pressure cell at 750 p.s.i. and 200 µl removed and stored at -70°C: Whole Cell lysate [Sample 2]. Whole cells and debris were then removed by two rounds of centrifugation at 5,000 rpm for 10 min, 200 µl was removed and stored at –70°C: Cell-Free Extract [Sample 3]. The supernatant was then centrifuged at 100,000 x *g* for 1 h. The resulting supernatant was frozen at –70°C: Cytoplasmic Fraction [Sample 4]. The pellet was then resuspended (with sonication) in 2.5 ml 50 mM Tris-HCl (pH 7.4) plus 5 mM $MgCl_2$, 300 µl was removed and stored at -70°C: Total Membranes [Sample 5]. To the remaining total membrane preparation Triton X-100 was added to a final concentration of 2% (v/v). This was mixed by sonication to partially solubulise the proteins, and then centrifuged at 100,000 x *g* for 1 hour. The supernatant was removed, frozen at -70°C and labelled Enriched Inner Membrane [Sample 6]. The pellet was then washed with 2.5 ml Tris-HCl (pH 7.4) and spun again for 1 hour at 100,000 x *g* before being resuspended in 1 ml Tris-HCl (pH 7.4) by sonication, this was the Enriched Outer Membrane fraction [Sample 7].

2.22.2 First Dimension - Isoelectric Focussing (IEF)

Approximately 100 µg protein was run in the first dimension. The quantities were estimated by SDS-PAGE and visualised by Coomassie Blue staining. 100 µg of protein was precipitated for 4 h with 4 volumes of ice-cold acetone (on ice) to remove excess salts. The proteins were recovered by centrifugation at 13,000 rpm in a benchtop centrifuge. After washing in 80 % (v/v)acetone the pellets were dried and resuspended by incubation for 30 min at 37°C to ensure complete solubulisation of proteins in 250 µl of IEF rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS,

20 mM DTT, 0.5% Triton X-100, 0.5 % Pharmalyte 3-10 [Amersham, Pharmacia], a few grains of Bromophenol Blue). Insoluble material was removed by centrifugation.

First Dimension, IEF, was run using 13 cm pH 3-10 non-linear Immobiline drystrip Immobilised pH Gradients (IPG) (From Amersham Pharmacia). Before IEF, the IPG strips must be rehydrated overnight in the presence of the protein using an Immobiline DryStrip Reswelling Tray as per manufacturer's instructions (Berkelman and Stenstedt, 1998). After rehydration, the strips were rinsed with distilled water and drained. The IEF was run using a Multiphor II cooled (20°C) horizontal gel tank system and a Drystrip aligner (Amersham Pharmacia) using the following protocol, programmed in gradient mode with the current check option switched off.

Step	Voltage (V)	Current (mA)	Power (W)	Time (h)
1	300	2	5	0:40
2	3500	2	5	1:30
3	3500	2	5	4:20
Total				6:30

After IEF, the strips are used immediately for the second-dimension or stored at -70°C.

2.22.3 Second Dimension - SDS PAGE

The second-dimension separates the focussed proteins by molecular mass. A Bio-Rad Protean II X-Cell gel system was used. The gels were essentially prepared in the same way as in section 2.19 except that 150 ml of gel mix was routinely used to pour four gels at one time and no stacking gel was used.

The 12 % SDS gel must be set before the IEF strips are equilibrated for the second-dimension.

2.22.4 IPG strip equilibration

Firstly the IEF strip was equilibrated in 10 ml Equilibration Buffer [(50mM Tris-HCl (pH 8.8), 6 M Urea, 30 % (v/v) Glycerol, 2 % SDS (w/v), Bromophenol Blue) with 10 $mgml^{-1}$ DTT added prior to use] for 15 min on a shaker. The second equilibration step was performed with the DTT substituted with 25 $mgml^{-1}$ iodoacetamide for a further 15 min. After equilibration, the strip was drained on filter paper, dipped in SDS running buffer and then placed on top of the SDS separating gel, between the plates. Size markers were soaked onto a piece of 3MM filter paper

and placed on top of the gel next to the strips. A solution of warm 0.5 % (w/v) agarose (dissolved in SDS running buffer) is poured in the top of the strip to seal the strip in place.

The voltage used for separation in the second dimension was:

Step	Voltage (V)	Current (mA)	Power (W)	Time (h)	mA/H
1	500	20	30	2:00	40
2	500	35	30	12:00	410
Total				14:00	436

After separation the gels were immediately stained or blotted.

2.22.5 Silver Staining

2DSDS-PAGE gels were Silver-stained using the following protocol:

1. Cover the gel with fixative (40% (v/v) ethanol and 10 % (v/v) glacial acetic acid) for 1 hour.
2. Wash the gel three times for 10 min in 10 % (v/v) ethanol.
3. Sensitize by a 20 min treatment with 2 % glutaraldehyde.
4. Wash three times for 10 min with distilled water.
5. Incubate for 20 min with Silver solution (A solution of $NaOH/NH_3$ [0.084 M/0.84 M respectively] was added to 10% (w/v) $AgNO_3$ during constant stirring until the precipitate dissappeared. The solution was diluted with water until the final concentration of $AgNO_3$ was 0.4% (w/v).
6. Wash the gel three times for 5 min with water
7. Develop in 0.0025 % (w/v) citric acid/0.00925 % (v/v) formaldehyde solution to the desired extent.
8. Development was stopped by rinsing with water several times.

All reactions were performed at room temperature on a rotary shaker.
Stained gels were scanned immediately on a BioRad 2D image scanner.

2.22.6 Western blotting of 2D-Gels

Western blotting of 2D-gels were performed using the Multiphor II Novablot Electrophoretic Transfer Unit. The following solutions were used:

Anodic Solution 1:

0.3 M Tris-base
20% (v/v) methanol
adjust to pH 10.4 with HCl

Anodic Solution 2:

25mM Tris-base
20 % (v/v) methanol
adjust to pH 10.4 with HCl

Cathodic solution:

4mM 6-Amino-n-hexanoic acid
20 % (v/v) methanol
adjust to pH 7.6 with HCl

Firstly, the Novablot plates were soaked in dH_2O for one hour prior to use. The anodic plate was placed into the Multiphor II unit and 6 filter papers, soaked in anodic solution 1, were stacked on top of this. On top of this were placed 3 filter papers soaked in anodic solution 2. Next a nitrocellulose membrane was cut to the same size as the gel and soaked in anodic solution 2. The gel was gently placed on top of the membrane, followed by 9 filter papers soaked in cathodic solution. The cathodic plate was then put in place and connected to the power pack.

The electro blotting was perfomed as follows:

Step	Voltage (V)	$mA/cm^2$2D-gel	Power (W)	Time (h)
1	500	0.8	30	1

After the protein transfer, the blotting procedure was carried out as described in section 2.20.

2.23 sMMO naphthalene assays

The activity of the soluble methane monooxygenase was routinely assayed at a qualitative level by the naphthalene assay (Brusseau *et al.*, 1990). Strains were grown on low-copper plates for 7-10 days before incubation for 30 min at 30°C in the presence of naphthalene crystals. 10 mg/ml tetrazotized *o*-dianisidine (Sigma) was then dropped onto the colonies. A positive result was noted by the development of a deep purple colour by the colony, and negative by no colour change (orange).

A similar procedure was followed for liquid cultures. 1ml of a 50 ml culture was incubated in an Eppendorf tube with 1 crystal of naphthalene for 30 min at 30° C before addition of 10 mg/ml tetrazotized *o*-dianisidine. Again the appearance of a purple colour was indicative of sMMO activity, although this was less strong in liquid cultures.

2.24 Copper reductase assay

This crude assay was developed from the methods of Georgatsou *et al.*, 1997 and Wunderli-Ye *et al.*, (1999). It relies on the fact that the copper (I) chelator bathocuproinedisulphonic acid (BCS) gives a red colour when complexed with cuprous ions. This can be measured in a spectrophotometer at 482 nm (extinction coefficient at A_{482} 12.25 $mM^{-1}cm^{-1}$ for the Cu(I)-BCS complex). *Methylosinus trichosporium* OB3b was grown on NMS medium with 20 % methane (v/v). 50 ml of mid-exponential phase cultures (OD_{540}~0.5) were harvested by centrifugation at 10,000 rpm in a Beckman JA20 rotor. The cell pellet was resuspended in Reductase Assay Solution (200 mM MOPS, 20 mM trisodium citrate and 2 mM $MgCl_2$ (pH 7.0)) to give an OD_{540} = 0.5.

10 ml (equivalent to $1x10^9$ cells) was placed in a sterile universal sealed with a size 41 suba-seal. These samples were then gassed with 20 % methane. BCS was added to a final concentration of 500 μM and copper sulphate at the concentrations indicated in the results section. Assay mixtures were then incubated at 30°C (without shaking) and 1 ml samples removed at the indicated time intervals and absorbance at 482 nm measured. Appropriate controls containing assay mixtures with no cells, and boiled cells were performed in all cases.

Example of copper reduction rate calculation:

The slope of the line generated by plotting A_{482} against time was calculated using the linear regression tool from the Microsoft Excel Package. This gives the increase in absorbance per minute.

Using the Beer-Lambert Law :

1. $A=Ecl$

A is absorbance, the figure used here represented the increase in absorbance per minute.

c is concentration of copper reduced in $mMmin^{-1}$ per 10^9 cells.

l is path length of cuvette, this is 1 cm.

E is the extinction coefficient expressed in $mMcm^{-1}$representing the absorbance of the Cu[I]-BCS complex at 482 nm. The value used here was 12.25 $mMcm^{-1}$.

If $A = 0.05\ min^{-1}$ and $l = 1$ cm and $E = 12.25\ mMcm^{-1}$ $A = Ecl$

2. Then $c = A/El$

$= 0.005/12.25$

$= 4.1 \times 10^{-4}\ mMmin^{-1}$ per 10^9 cells

$= 0.41\ \mu Mmin^{-1}$ per 10^9 cells

$= 0.41\ nmolsmin^{-1}$ per 10^9 cells

CHAPTER 3

Transcriptional analysis of the *pmo* gene clusters from *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M

3.1 Introduction

In recent years the genes encoding the particulate methane monooxygenase (pMMO) enzyme have been cloned and sequenced from three methanotroph strains: *Methylococcus capsulatus* Bath (Semrau *et al.,* 1995; Stolyar *et al.,* 1999), *Methylocystis* sp. strain M (Gilbert *et al.,* 2000) and *Methylosinus trichosporium* OB3b (Gilbert *et al.,* 2000; McDonald, personal communication). The genes encoding the pMMO enzyme (*pmo*) are present in duplicate copies in the order *pmoCAB* in all three organisms. The duplicate copies have a very high degree of identity between them, as exemplified by *Methylococcus capsulatus* Bath which has only 13 nucleotide sequence differences over 3,183 bp (Stolyar *et al.,* 1999).

Analysis of the genes adjacent to the *pmoCAB* cluster of *Methylosinus trichosporium* OB3b revealed that the second copy has two open reading frames upstream of *pmoC* (Figure 3.1). The derived amino acid sequence of one ORF has 50% identity at the amino-acid level to *folC,* a dihydroxypteroate synthase from *Methylobacterium extorquens,* and the other 49% amino acid identity with *hppK*, a 7,8,Dihydroxymethylpterin-pyrophosphokinase from *Sinorhizobium meliloti* (Figure 3.1) (I. McDonald, personal communication). Both of these genes are involved in the biosynthesis of folate, a key component of methylene tetrahydrofolate, a central intermediate of the serine cycle which is known to be the route for formaldehyde assimilation in type II methanotrophs (Chistoserdova and Lidstrom, 1997). They are thus likely to be important in C_1-metabolism in *Methylosinus trichosporium* OB3b (Hanson and Hanson, 1996), indicating that *pmoC* copy 2 may lie within a C1 metabolism cluster. Indeed, the *folC* gene from *Methylobacterium extorquens* AM1 lies in an operon with a *hppK* homologue within a large cluster of genes implicated in C_1-metabolism (Chistoserdova and Lidstrom, 1997). Upstream of the sequenced copy of *pmoC* from *Methylocystis* sp. strain M lies part of an open reading frame named *orfX,* this has 56% identity to cytochrome c_{551} peroxidase from *Pseudomonas aeruginosa.* However, none of the surrounding genes discovered so far are implicated by homology searches in transcriptional regulation.

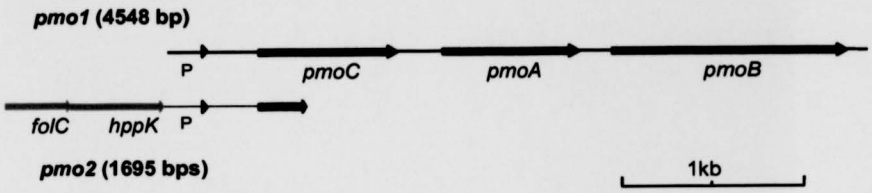


Figure 3.1 Genetic organisation of the sequenced regions of the two copies of *pmoCAB* operon from *Methylosinus trichosporium* OB3b. Copy 1 is the fully sequenced operon as shown in Gilbert *et al.*, (2000). Putative promoter regions are denoted by P (produced with the permission of Dr I. McDonald).

In a recent study by Stolyar *et al.* (2001), the transcriptional start sites and associated putative promoters for the two *pmoCAB* clusters from *Methylococcus capsulatus* Bath were identified. Both copies possess putative promoters with a high degree of identity to the *E. coli* σ^{70} –10 and –35 promoter consensus sequences (Wösten, 2000). Immediately upstream of each promoter sequence lies a highly conserved region (CCTGCGTCAAAATCt/aCTCAg/tATTTTTC), which the authors propose to be a putative regulatory sequence. Their study also included the construction of chromosomal *xylE*-promoter fusions for the two copies of the *pmo* cluster, which revealed that the copy 2 promoter is expressed at twice the level of the copy 1 promoter under normal growth conditions (5 μM copper) (see section 1.5.1 for further details).

In methanotrophs containing both the pMMO and sMMO enzyme systems, the pMMO is expressed under high copper to biomass ratios and the sMMO at low copper to biomass ratios (Stanley *et al.*, 1983, Nielsen *et al.*, 1996, 1997). The promoter sequences for the sMMO encoding operon (*mmo*) of both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath have been identified by primer extension (Nielsen *et al.*, 1996,1997). Upstream of *mmoX Methylosinus trichosporium* OB3b lies a transcriptional start site corresponding to a σ^{N} promoter. Interestingly, another putative start site and promoter were found between *mmoX-mmoY* but it is unclear whether this is a functional promoter (Nielsen *et al.*, 1997). However, 5' of the *mmoX* gene in *Methylococcus capsulatus* Bath lies a promoter with high identity to the consensus sequence for the –24 motif of a σ^{N}

promoter but not the –12 motif. Thus, it appears the regulation of the *mmo* cluster may be different in these two methanotrophs.

To further characterise the newly sequenced *pmoCAB* clusters from *Methylocystis* sp. strain M and *Methylosinus trichosporium* OB3b an investigation of the transcript start sites was performed using primer extension and RT-PCR techniques, in order to reveal further insights into the regulation of this unique system.

3.2 Primer extension analysis of *pmo* gene cluster from *Methylocystis* sp. strain M

Primer extension was carried out in collaboration with Bettina Gilbert and Allan K. Nielsen and was published during the course of this project (Gilbert *et al.*, 2000). This method allows the location of transcriptional start sites.

Total RNA was extracted from high-copper (3 μM) grown batch cultures of *Methylocystis* sp. strain M and *M.trichosporium* OB3b and from a chemostat culture (NMS containing 1 mgl^{-1} $CuSO_4$) of *M.trichosporium* OB3b as described in Section 2.3.4. These cultures were tested for sMMO activity using the naphthalene assay (Brusseau *et al.*, 1990) and shown not to oxidise naphthalene, and were therefore assumed to be expressing pMMO. These RNA preparations often contained DNA (Figure 3.2) which was removed by DNAse digestion (as described in section 2.17).

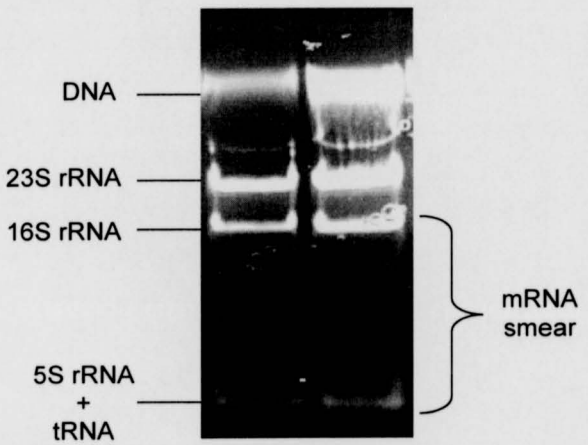


Figure 3.2 Total RNA from *Methylosinus trichosporium* OB3b
3 μl of total RNA extracted from a 50 ml batch cuture (OD_{540}=0.5) was loaded per lane on a 1 % TBE-Agarose gel.

Table 3.1 Primers used in primer extension experiments. Position numbers refer to numbering with respect to sequence accession numbers AF186586 for *Methylosinus trichosporium* OB3b (labelled O) and AF186587 for *Methylocystis* sp. strain M (labelled M).

Primer	Position in respective *pmo* cluster	Sequence (5' to 3')
O1	295-278	GCG CAG AAC GAG CGC TCC
O1A	118-101	TGG CGC CGC AAA ATG ACG
O1B	373-355	CCT CCC GTC GCG CCG ATC C
O1C	415-398	GGT TAC GTG AAA TAT GC
O1D	335-319	GTT GTT CTT CTT TGT GC
O1E	316-300	GAC GAA GTC CCC AAA GC
O1F	304-286	AAA GCC GCC GCG CAG AAC G
O2	514-497	TGT TGT TAC GCT CAT CTC
O2A	487-463	AAG TGC TCC CAG GGA TTT CGA GGC
O3	1529-1502	CTT CGA TGT AAA CAT CAC
O4	2481-2465	TCG CCA GTT CGG CCA TTC
M1	651-634	GGA CGA ACA CCG ACG TCG
M1A	680-663	ACA TCC AAT AAG GCC GCC
M2	891-874	CAG CCG TGC TAG TCG TCG
M3	1982-1965	CCG CTT TTC GAT TGT GAC
M4	2867-2851	GCG GCG AGC TTG ACT AGC

The position of the primers used in these experiments is listed in table 3.1 and shown schematically in figure 3.3. These primers targetted the regions upstream of *pmoC*, the intergenic regions between *pmoC-pmoA* and *pmoA-pmoB*.

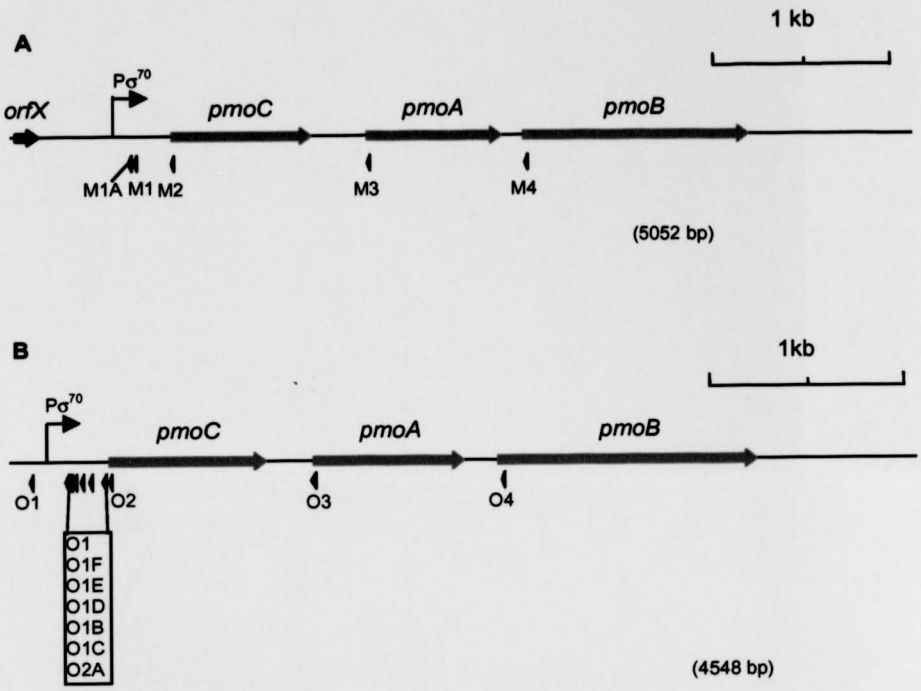


Figure 3.3 Genetic map of the *pmo* clusters from *Methylocystis* sp. strain M (A) and *Methylosinus trichosporium* OB3b (B). Primer locations are shown by blue arrowheads. Putative promoter regions ($P\sigma^{70}$) are also indicated. Exact primer positions are listed in Table 3.1

Two putative 5' transcript ends were identified 5' of *pmoC* in *Methylocystis* sp. strain M. using primer M1 (Figure 3.4). The stronger of the two signals mapped at A_{574}. Directly upstream of A_{574} lies a promoter with high identity to the σ^{70}-promoter consensus sequence : TTGACA-N_{17}-TATAAT-N_6-X (Figure 3.5) (Wösten, 2000). The second, weaker, signal mapped to T_{586} and also contained a σ^{70}-promoter consensus sequence in its upstream (5') region. In the case of A_{574} the promoter matched the consensus at 5/6 positions of the –35 motif and 3/6 in the –10 motif, for A_{586} the match was 4/6 and 3/6 respectively. However, sequence was not available for both copies of the operon in this region so it is not clear if these promoters are present on one or both copies of the *pmo* cluster. Primer extension using the primer M1A (located 29 bps 3' of M1) was performed to confirm the location of these two putative transcriptional start sites and gave identical results. Therefore, the mRNA is initiated 300 bp 5' of the start codon for *pmoC* from at least one σ^{70}-type promoter.

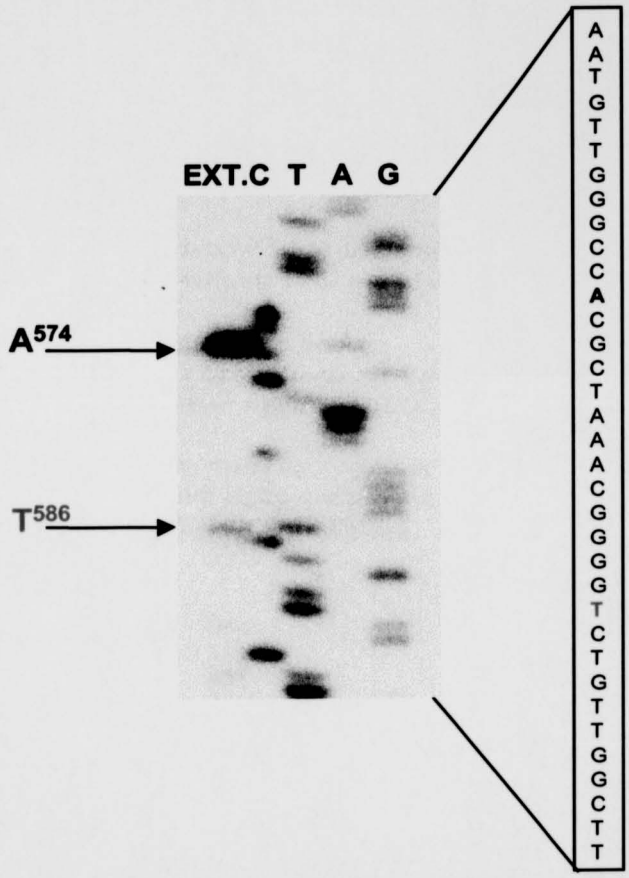


Figure 3.4 Primer extension analysis showing transcriptional start sites 5' of *pmoC Methylocystis* sp. strain M. Transcriptional start sites are shown in Green (A_{574}) and Red (T_{586}). Numbering corresponds to accession number AF186587. Ext. refers to primer extension reaction using primer M1. Sequencing reactions were run alongside in lanes C, T, A, G.

```
Consensus:      TTGACANNNNNNNNNNNNNNNNNTATAATNNNNNNN
strain M :537   TTGTCATTATGTTGGTGAGAGGGAATGGTTGGGCCACGCTAAACGGGGTCTGTTGGC  594
OB3b     :183   TTGTCATTGAAGACGCGTACGGAAATGGTTGCGGCTCGCCGGACGAGGGTTTTCGGC  240
                -35 MOTIF                -10 MOTIF      +1
```

Figure 3.5 Alignment of putative promoter regions of *pmo* gene clusters of *Methylocystis* sp. strain M and of *Methylosinus trichosporium* OB3b. Putative promoter regions are highlighted by Green (A_{574}) and Red (T_{586}) lines above the sequence. The *E.coli* σ^{70} promoter consensus is also shown. Numbering corresponds to accession numbers AF186587 and AF186586 respectively.

The primers targetted to the intergenic regions of the *pmoCAB* operon gave weak signals in all cases (data not shown). Primer M2, located within the 5' end of *pmoC* gave two signals at C_{827} and T_{828}. The intergenic primers M3 and M4 gave products at G_{1916} and T_{2789}. All of the products for M2, M3 and M4, were very weak in comparison to that of A_{574} and none of them had putative promoter sequences upstream of putative transcriptional start sites. These 5' mRNA ends probably originated from processing of a longer transcript initiating upstream of *pmoC*.

Primer extension analysis of the *pmoCAB* operon from *Methylosinus trichosporium* OB3b was also performed. Despite repeated attempts and the use of all primers listed in table 3.1, the identification of any consistent 5' mRNA ends from RNA extracted from this organism was unsuccessful.

To attempt to overcome any possible problems with secondary structure elements preventing the reverse transcription step from proceeding to completion the denaturing temperature was increased to include a range of temperatures from 75°C-95°C and the extension temperature increased to 50°C. However, all attempts resulted in either no products or a smear in the extension product lanes (Data not shown). This result was very surprising because inspection of an alignment of the region upstream of *pmoC* between *M. trichosporium* OB3b and *Methylocystis* sp. strain M revealed striking similarities (Figure 3.5). The promoter corresponding to A_{574} from *Methylocystis* sp. strain M is clearly present in the sequence upstream of *pmoC* in *M.trichosporium* OB3b (underlined on both copies of the cluster, Figure 3.6). But, interestingly the promoter corresponding to A_{586} is absent from both copies of the *M. trichosporium* OB3b *pmo* cluster.

```
        +  + +          +   ++  ++++++       ++++++  ++  ++++++++++++++++++++++++++++++++++++++++
copy 1-CTGACAGGCTTGTGCTTCCCATTCGATAGATTACTTTCTCCTACCCTATCGGATATTGTCATTGAAGACGCGTACGGAAATGGTTGCGGCT
copy 2-TTATCGGCAGACGAAGAACTGCTCATTAGATTGTCACAGCCTACCTGATTAGATATTGTCATTGAAGACGCGTACGGAAATGGTTGCGGCT
Str. M-CCACTGGGGTTGTTTGGACTATTCGATAGACAGTTGAATGCTACCCTGAGGGGTATTGTCATTATGTTGGTGAGAGGGAATGGTTGGGCCA
             *          *   **  ****         *****      * **********      * *   ** ******** * *
```

Figure 3.6 Alignment of putative promoter regions of *pmo* copy 1 and copy 2 from *Methylosinus trichosporium* OB3b with *Methylocystis* sp. strain M. The major transcriptional start site (A_{574}) identified 5' of the *pmo* operon of *Methylocystis* sp. strain M is highlighted in red. Identities between the two copies of *pmo* in *M. trichosporium* OB3b are denoted by a plus-sign (+). Those nucleotides conserved between all three sequences are denoted by an asterisk. The putative promoter and start sites identified for *M. trichosporium* OB3b by identity with *Methylocystis* sp. strain M are underlined. The promoter identified in *pmo* copy 2 of *M. trichosporium* OB3b by similarity to *E.coli* consensus is highlighted by blue text.

A comparison of the upstream (5') sequences of the two copies of the *pmo* cluster from *M. trichosporium* OB3b revealed that the sequences of the two copies begin to diverge 4 bps 5' of the putative promoter identified by identity with the corresponding region from *Methylocystis* sp. strain M (Figure 3.6). Therefore, it seemed likely that the promoter lies within this region. It was possible that this promoter-like sequence is not functional in *M. trichosporium* OB3b, but it is more likely that the primer extension reaction did not work for unknown reasons.

3.3 Confirmation of *pmo* promoter region from *Methylosinus trichosporium* OB3b by RT-PCR

In order to confirm the location of the putative promoter upstream of the *pmoCAB* cluster from *M. trichosporium* OB3b, a method was developed based on RT-PCR. RT-PCR involves the reverse transcription of RNA into complementary DNA (cDNA), which is used as a template for PCR. In this way, the presence or absence of a gene-specific RNA species can be established, thus indicating transcription of that gene. However, if the transcriptional start site of the RNA molecule (and therefore cDNA produced by reverse transcription) occurs 3' of the position at which the forward primer for PCR would normally bind, no product should be seen even if the gene was transcribed.

Based on this principle, a method was developed whereby cDNA was produced from a reverse primer designed to bind to the mRNA at the 5' end of *pmoC* (O2). This cDNA molecule should extend back from *pmoC* to the 5' end of the *pmo* specific transcript. The cDNA produced was used as template for the PCR step. The reverse primer for this reaction was O2. However, separate reactions were set-up using different forward primers: pmo-35F, located 5' of the proposed transcriptional start site; pmo+7F, located 3' of the proposed transcriptional start site (exact locations shown in Figure 3.8B). In theory the primer 5' of the transcriptional start site (pmo-35F) should give no product and the other (pmo+7F) a *pmo* specific product. This would then confirm/ reject the proposed transcriptional start site (summarised graphically in Figure 3.7).

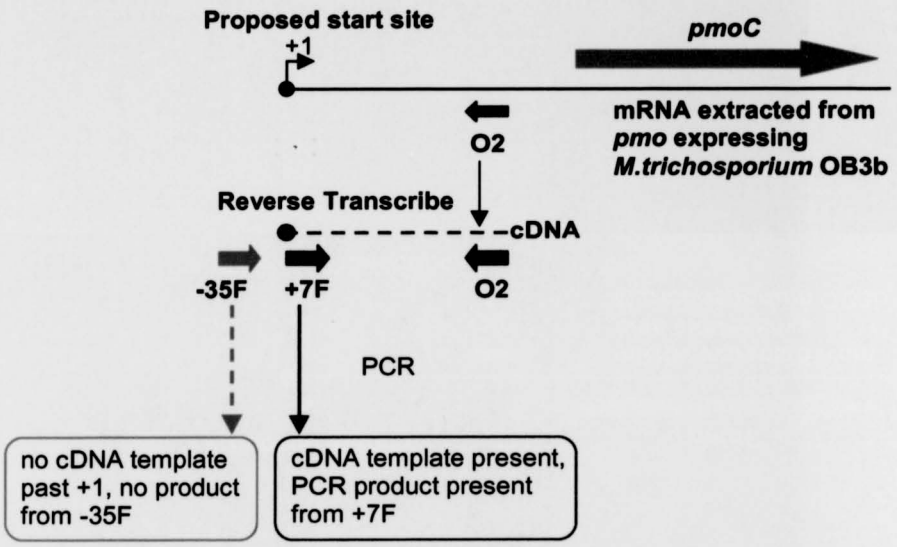
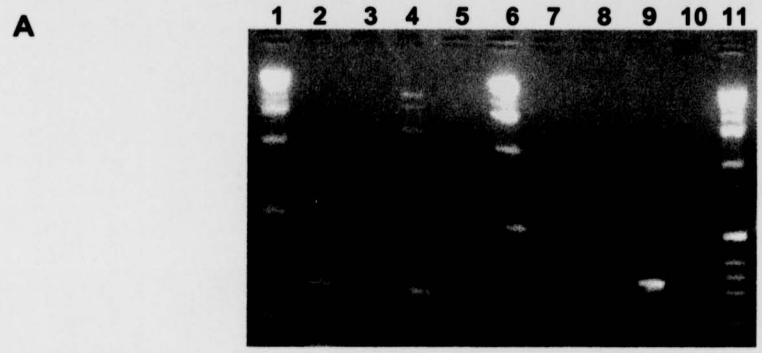


Figure 3.7 Schematic representation of RT-PCR method developed for localisation of transcriptional start site for the *pmo* cluster from *Methylosinus trichosporium* OB3b. mRNA was reverse transcribed from primer O2 and the cDNA used as a template for PCR using primers O2 (listed in table 3.1) and pmo+7F or pmo-35F (figure 3.8b). If primer pmo-35F lies 5' of the transcriptional start site there will be no cDNA template to which it can bind and no product would be expected using this primer.

The results of this experiment are shown in Figure 3.8A. They clearly show that there is an RT-PCR product using the pmo+7F primer and none for the pmo-35F primer. In all cases, RNA-negative and DNA-positive PCR control reactions were carried out.



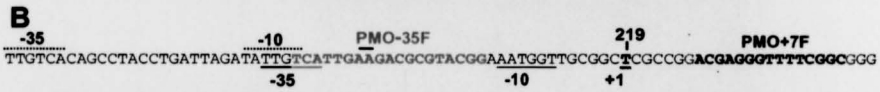


Figure 3.8 RT-PCR analysis of proposed transcriptional start site for *pmo* operon of *Methylosinus trichosporium* OB3b.
A 2 % TBE-Agarose gel of RT-PCR. Lanes: 1 1kb marker, 2 cDNA, 3 RNA, 4 OB3b DNA, 5 No DNA, 6 1kb marker, 7 cDNA, 8 RNA, 9 DNA, 10 No DNA, 11 1kb marker. Lanes 2-5 used primer pmo+7F and O2. Lanes 7-10 used primer pmo-35F and O2. These results were verified using independent triplicate cultures.

B Proposed promoter region upstream of *pmoC* in *M. trichosporium* OB3b. Promoter motifs and transcriptional start site proposed by sequence comparison from *Methylocystis* sp. strain M are underlined. A second proposed promoter and transcriptional start site present on copy 2 only are overlined..Primers used are coloured in red and green for pmo-35F and +7F respectively.

These data confirm that the transcriptional start site for the *pmoCAB* cluster of *M. trichosporium* OB3b lies within the 19 bp region between these two primers and probably at T_{219}. This is futher supported by the evidence from the promoter region alignments between *Methylocystis* sp. strain M and *Methylosinus trichosporium* OB3b showing that the promoter motif corresponding to A_{574} is present at 100% identity in *M. trichosporium* OB3b (Figure 3.6). Interestingly, an alignment between the two copies of the *pmo* clusters from *M. trichosporium* OB3b in this region reveals that the copies of the two sequences begin to diverge 4 bp 5' of the putative promoter sequence (Figure 3.6), and then diverge completely at 25 bp. This evidence adds

further strength to the importance of this region of DNA and more credence to the identification of the putative σ^{70}-type promoter.

3.4 Restriction analysis of the promoter regions of both *pmo* clusters of *M.trichosporium* OB3b

The sequences of the promoter regions from the two copies of the *pmo* cluster in *M. trichosporium* OB3b are almost identical but show divergence between the two copies 4 bp 5' of the –35 promoter motif, and complete divergence at 25 bp (Figure 3.6). In the region between the proposed transcriptional start site and the ATG codon of *pmoC* there apeared to be two differences, one of these: T_{384}; causes a restriction-site difference of *Hinf*I on copy 1 and *Mlu*I on copy 2, where a G is present at position 384. Such a difference would allow a crude semi-quantitative assessment of copy-specific transcription by the formation of RT-PCR products followed by their restriction digestion with both of these enzymes and comparison between cut and uncut products. The RT-PCR digests could be compared to standards with known ratios of copy 1 and copy 2 PCR products.

However, digestion of the 322 bp PCR product amplified using primers O2 (Table 3.1) and pc6F (CGGCGCCATATATAAACAGC) from chromosomal DNA of *Methylosinus trichosporium* OB3b resulted in fragments of 130 and 190 bp from an *Mlu*I digest and only a full length PCR fragment from a *Hinf*I digest (Figure 3.9). This suggested that the true sequence of this region contained a G at position 384, thus giving an *Mlu*I site and not a *Hinf*I site in both copies. This discovery meant that the crude analysis suggested above was impossible as there were no differences in the region sequenced on both copies.

Figure 3.9 Restriction of *pmo*-promoter region from *M. trichosporium* OB3b. PCR was performed using the primers O2 and pc6F (see text). Digests were performed using 10µl PCR product, in a total volume of 20µl with the appropriate restriction buffer for *Mlu*I and *Hinf*I.

3.5 Discussion

The *pmo* cluster of *Methylocystis* sp. strain M contains tandem overlapping promoter sequences originating from two separate transcriptional start sites. The weaker of the two signals could be a false positive created by premature termination of the reverse transcription step. However, it seems more likely that both of these putative transcriptional start sites and promoters are real and that they both play a role in the transcription of these genes. This is supported by recent evidence showing that under different growth conditions *Methylococcus capsulatus* Bath predominantly expresses one of the copies of *pmo* over the other, indicating differential regulation of transcription from these two promoters (Stolyar *et al.*, 2001, reviewed in more detail in section 1.5.1). Further studies of *pmo* transcription in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b revealed that although chiefly expressed at high copper:biomass ratios, there are detectable levels of *pmo* transcripts even on low copper media (Stolyar *et al.*, 2001, This study, chapter 4).

It is therefore possible that the tandem promoters from *Methylocystis* sp. strain M may be used under different conditions, one allowing a basal level of transcription under low copper conditions and one inducible at high copper concentrations. Several well-characterised examples of tandem promoters involving differential expression from σ^N and σ^{70}-type promoters exist, such as the *glnA* promoter system from *Escherichia coli* (Albright *et al.*, 1989; Merrick and Edwards, 1995) and the XylS/XylR promoters from the *Pseudomonas putida* TOL plasmid (Marques *et al.*, 1998). Other operons are regulated by two sigma factors, such as the *fliA* and *fliL* class II flagellar genes from *E. coli* where σ^{28} and σ^{70} compete for promoter binding (Liu and Matsumura, 1996). The presence of tandem σ^{70} promoters in *Methylocystis* sp. strain M appears to be unusual. However, Gräfe *et al.*, (1996) showed that the streptokinase (*skc*) gene from *Streptococus equisimilis* H46A is transcribed from two overlapping σ^{70}-type promoters whose activity is stimulated by up to 30-fold by a 202 bp upstream sequence. But, transcription from these promoters appears to be mutually contributive to maximal expression and not differentially regulated (Gräfe *et al.*, 1996). Differential expression does occur in the case of the tandem σ^{70} promoters which exist upstream of the *ccpA* gene from *Lactobacillus plantarum*, here transcription occurs only from P1 in the presence of glucose and from P2 in the

presence of ribose. However, the *ccpA* gene product itself acts to repress transcription from P2 in the presence of glucose via a catabolite responsive element, which overlaps the P2 transcriptional start site (Muscariello *et al.*, 2001). Perhaps the most closely related genes to the *pmoCAB* cluster are the *amoCAB* genes from nitrifying bacteria, such as *Nitrosomas europaea* (Holmes *et al.*, 1995). In addition to possessing duplicate copies of the *amoCAB* operons, primer extension studies revealed the presence of tandem σ^{70}-type promoters located 5' of both copies of *amoC* in *Nitrosomanas europaea* (Hommes *et al.*, 2001). Hommes *et al.*, (1998) showed that deletion of either copy of *amoA* resulted in an increase in transcription from the remaining copy of *amoA* but that deletion of $amoA_1$ resulted in a strain with only 65% of wild-type transcription levels, indicating that transcription from the two copies occurred at different levels. Hommes *et al.*, (2001) confirmed this theory by showing that transcription from P1 was dominant under ammonia sufficiency and occured from P2 under ammonia limitation. However, the promoter sequences are identical from both copies, indicating that this differential expression may be modulated via sequences 5' of these promoters where the sequences of the two copies diverge (Hommes *et al.*, 2001). It may be possible to determine the roles of the two tandem promoters 5' of *pmoC* in *Methylocystis* sp. strain M by performing primer extension on RNA extracted from cells grown on varying concentrations of copper. An examination of the relative abundance of the primer extension products from each promoter may reveal whether they respond differently to copper ions. It would be interesting to know if both, or just one, of these promoters is present on the second copy of the *pmo* cluster. However, in the absence of DNA sequence for both copies of the *pmo* cluster of *Methylocystis* sp. strain M, this remains unanswered.

The transcriptional start site for the *pmo* cluster from *M. trichosporium* OB3b proved harder to identify. A high degree of identity with the promoter region of *Methylocystis* sp. strain M. allowed the design of an RT-PCR experiment which enabled the localisation of the start site. The RT-PCR method developed here could prove useful in the localisation of a start site to a particular region of DNA, which can then be examined by primer extension. This method can also be used to examine whether a particular transcript bridges the gap between two genes (Franke *et al.*, 2001).

Sequence analysis of the promoter region of *Methylosinus trichosporium* OB3b revealed an exact match for the A_{574} specific promoter from *Methylocystis* sp. strain M, but did not contain a match to the T_{586} promoter. This indicates that regulation of the *pmo* clusters from these two organisms may be subtly different. Interestingly, the sequence 5' of *pmo* copy 2 from *M. trichosporium* OB3b has a match for another σ^{70}-type promoter which overlaps the promoter identified by identity with that identified *Methylocystis* sp. strain M (figure 3.6). The RT- PCR method used here does not allow confirmation or rejection of an mRNA start site corresponding to this promoter. These results do however confirm that the *pmo* genes of *M. trichosporium* OB3b are transcribed from at least one σ^{70} promoter 5' of *pmoC*.

Northern analysis of the *pmo* operon of *M. trichosporium* OB3b by Nielsen *et al.,* (1997) had previously revealed the presence of a *pmo*-specific transcript of approximately 4 kb from cells grown in high copper medium. The discovery of a promoter 5' of *pmoC* in this study, coupled with the absence of intergenic primer extension products for *M. trichosporium* OB3b and *Methylocystis* sp. strain M agrees well with this observation. Taken together the evidence strongly suggests that the *pmo* genes are transcribed as a 4 kb polycistronic transcript from this promoter.

It is tempting to speculate regarding the possibility of differential regulation between the two *pmo* clusters in *M. trichosporium* OB3b as seen by Stolyar *et al.,* (2001) in *Methylococcus capsulatus* Bath. Future investigations should focus on the role of the second putative promoter in the region where the two copies of the *pmo* cluster from *M. trichosporium* OB3b diverge and may provide a better understanding of their regulation. However, in the absence of gene fusions or copy-specific Northern blots it is not possible at present to draw any conclusions regarding differential expression of the two copies of *pmo* in *M. trichosporium* OB3b.

Whilst both *Methylocystis* sp. strain M and *M. trichosporium* OB3b promoter regions possess high identity with σ^{70} promoters, neither contain the proposed regulatory sequences present in the corresponding region of DNA in *Methylococcus capsulatus* Bath (Stolyar *et al.,* 2001). Analysis of an alignment of the upstream region of *pmo* copy 1 and *pmo* copy 2 from *M. trichosporium* OB3b and *Methylocystis* sp. strain M revealed two conserved regions 5' of the promoter sequence: CTACC and TAGA, which may be important in regulation (Figure 3.6). The divergence of the two sequences 5' of the –35 element of the promoter also

suggests that any regulatory elements involved in activation/repression may lie in the stretch of DNA from the promoter to the ATG-codon of *pmoC*.

The data from *M. trichosporium* OB3b and *Methylocystis* sp. strain M clearly show that the regulation of the *pmo* gene clusters in these two type two methanotrophs is very different from that of the *mmo* (encoding the soluble methane monooxygenase). The *mmo* operons from these two organisms possess σ^N-type promoters 5' of the first gene in the operon. This was confirmed by primer extension for *M. trichosporium* OB3b (Nielsen *et al.*, 1997) and by sequence similarity to σ^N-consensus for *Methylocystis* sp. strain M (McDonald *et al.*, 1997). The σ^N-type promoters always require transcriptional activators to aid in the initiation of transcription. Their absence from the *pmo* operons of both *M. trichosporium* OB3b and *Methylocystis* sp. strain M suggests a contrasting mechanism of regulation between the two MMO gene clusters.

It is well established that the *pmo* and *mmo* operons in both *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M are regulated by copper ions. In recent years there has been an increase in the number of reports in the literature regarding copper regulated systems. To date, three principal modes of regulation have emerged. The availability of sequences covering the promoter regions of the *pmo* clusters of *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M enabled their similarity to known copper-regulated promoters to be assessed.

The first mode of copper-regulation to be studied in detail was the *copRS* controlled *copABCDRS* copper resistance operon from *Pseudomonas syringae* (Cooksey, 1994, Mills *et al.*, 1993, 1994). Since its discovery, two similar systems have been isolated from *E.coli*, the plasmid-borne *pcoABCDRSE* copper resistance operon (Brown *et al.*, 1995; Rouch & Brown, 1997) and recently the *cusCFBA* chromosomally encoded operon (Munson *et al.*, 2000). In all three cases the copper operon is controlled by a two-component regulatory system, encoded by *copRS*, *pcoRS*, and *cusRS* respectively. All of these operons share a conserved palindromic 'copper-box' sequence motif which overlaps their promoters (Rouch & Brown, 1997; Munson *et al.*, 2000). The newly identified promoter regions of both *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M do not appear to contain a 'copper box' promoter.

The second system, also discovered in *E.coli* is the *cueR* (Cu Efflux Regulator), which is a member of the *merR* family of transcriptional regulators (Stoyanov *et al.*, 2001, Outten *et al.*, 2000, Petersen & Møller, 2000). The CueR protein has been shown to regulate *cueO* (encoding multi-copper oxidase) and *copA* (encoding a membrane-bound copper-transport ATPase) genes (Stoyanov *et al.*, 2001, Outten *et al.*, 2000). The promoters of this family are characterised by having an extended spacer (19 bps) between the –10 and –35 regions and are often involved with metal gene regulation (Brocklehurst *et al.*, 1999). These regulators activate transcription after binding at a site in this spacer region initiate transcription by a mechanism proposed to involve bending of the DNA bringing the (-10 and -35) promoter regions onto the same face of the DNA helix. The *pmo* promoters identified here have a spacer region of 17 bp, indicating they are unlikely to be under the control of a MerR type regulator.

The third, and most well characterised system is that of the *copYZAB* operon of *Enterococcus hirae*. This copper homeostasis operon is controlled by the action of the CopY repressor protein (a member of the LysR family), which allows derepression of *copYZAB* transcription in the presence of copper (Strausak and Solioz, 1997). It binds to the DNA at two sites, one spanning the –10 element of the promoter and one between this and the ATG codon of *copY* (Wunderli-Ye and Solioz, 1999). Once again, there is no match between this binding site and the sequence 5' of *pmoC* in both *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M.

The cognate transcriptional regulators are often located in close proximity to the operon on which they act. However, the absence of such genes in the regions surrounding the *pmo* operons of both *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M means that transcriptional regulation of the *pmo* clusters is governed by a novel mechanism.

Based on the available evidence, it appears that the regulation of the *pmo* operons of both *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M occurs via an unknown mechanism. This would employ an as yet undiscovered regulator which interacts with the σ^{70}-promoter. The majority of σ^{70} promoters are under the control of repressors (Collado-Vides, 1991). It is therefore tempting to speculate that the *pmo* operon is at least partly repressed in the absence of copper.

The divergence of the two copies 5' of the –35 motif of the *pmo* operon of *M. trichosporium* OB3b favours the involvement of a repressor as activators rarely bind to DNA overlapping the –10 region or further downstream (Collado-Vides, 1991). However the conserved motifs 5' of the promoters in both organisms do not allow us to dismiss the involvement of an activator here.

The results presented here suggest several further lines of investigation towards understanding the complex regulatory mechanisms involved with the *pmo* operon. Firstly, the importance of the two promoters from *Methylocystis sp.* strain M can be investigated using primer extension. Secondly, the cloning and sequencing of the upstream region from *pmoC* in *Methylocystis* sp. strain M would establish which promoters were present on each copy. Thirdly, the construction of full-length and truncated copy-specific promoter-fusions, similar to the *xylE* constructs used by Stolyar *et al.* (2001)(see section 1.5.1), to examine differential expression of the two copies of *pmo*. The construction of fusions using progressively truncated promoter fragments would also allow an investigation of the role of the conserved upstream sequences. Unfortunately, these experiments were not possible given the time-scale of this study.

The primer extension data presented in this chapter confirm that the three genes of the *pmoCAB* cluster are transcribed from σ^{70}-type promoters upstream of *pmoC* in *Methylocystis* sp. strain M and *Methylosinus trichosporium* OB3b. These data also confirm that the *pmoCAB* cluster is transcribed as one large polycistronic mRNA molecule. This work has provided further insight into this novel copper regulated system. It conclusively shows that the regulation of *mmo* and *pmo* operons are fundamentally different from each other and may reveal a novel mechanism of copper-mediated regulation, which should provide several years of work for future researchers.

Chapter 4

Analysis of the expression of *pmo* and *mmo* transcription by RT-PCR during growth of *Methylosinus trichosporium* OB3b on methane and methanol

4.1 Introduction

Stanley *et al.* (1983) discovered that the levels of copper in the medium controlled expression of the two methane monooxygenase enzymes, from *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b. However, until relatively recently, little was known about the details of this 'copper switch' at the transcriptional level. Nielsen *et al.*, (1996, 1997) showed that *mmo* transcription was completely inhibited by copper ions in these two organisms (See section 1.5.1). However, recent results presented by Stolyar *et al.*, (2001) suggest that low levels of *pmo* transcripts persist in the presence of high and low concentrations of copper in the medium from *Methylococcus capsulatus* Bath. The first aim of this study was to assay in a qualitative manner for *pmo*-specific transcripts from *Methylosinus trichosporium* OB3b.

Methylosinus trichosporium OB3b is also capable of growth using methanol as a sole carbon and energy source. Little is known about expression of the sMMO and pMMO enzymes in the presence of methanol. Several studies indicate that the pMMO enzyme is present in cells grown on methanol (Finch, 1997; Dalton *et al.*, 1984; Section 1.5.2). However, the transcription of the *pmo* and *mmo* operons from *Methylosinus trichosporium* OB3b under such conditions has never been examined. Thus, the second aim of this study was to examine transcription of the *mmo* and *pmo* operons at a qualitative level when cells were grown in the presence of methanol.

4.2 Transcription of methane monooxygenase genes during growth on methane: effects of copper ions

It is well established that *mmo* transcription is abolished when *Methylosinus trichosporium* OB3b is grown in the presence of high concentrations of copper (Nielsen *et al.*, 1997). However, recent work by Stolyar *et al.*, (2001) with *Methylococcus capsulatus* Bath has shown that *pmo* transcripts are present, albeit at low levels, under all copper ion concentrations tested. The aim of the work presented in this section was to determine qualitatively, by RT-PCR, when the *pmo* genes of *M. trichosporium* OB3b were transcribed, with respect to copper levels in the growth medium. Northern blotting by Nielsen *et al.* (1997) showed that the *pmoCAB* operon is transcribed as a single 4 kb transcript in *M. trichosporium* OB3b. In this study, RT-

PCR was performed using primers specific for the region upstream of *pmoC* in order to achieve the highest levels of sensitivity possible (Figure 4.1).

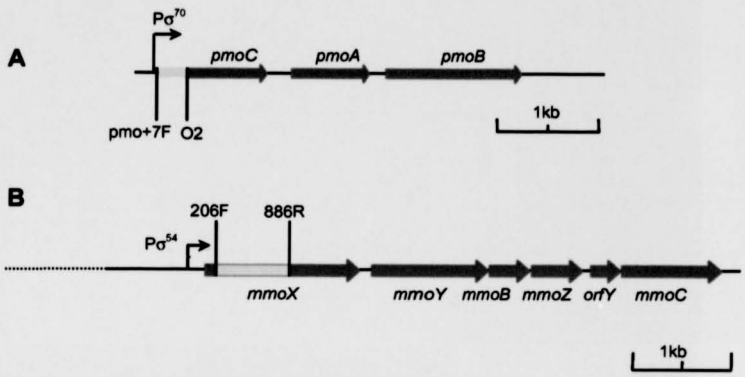


Figure 4.1 Genetic organisation and location of primers used for analysis of *pmo* (A) and *mmo* (B) operons. A: Primers pmo +7F (GACGAGGGTTTTCGGCG) and O2 (TGTTGTTACGCTCTCTC) were used to amplify the region from the putative promoter region to the start of *pmoC*, nucleotides 225 – 497 (numbered according to Gilbert *et al.*, 2000). **B:** Primers 206F (ATCGCBAARGAATAYGCSCG) and 886R (ACCCANGGCTCGACYTTGAA) were used to amplify the region from nucleotides 206 – 886 within *mmoX* (numbered from the start of *mmoX*).

Total RNA was extracted from triplicate batch cultures (50 ml) grown on NMS media containing high (5 μM) and low (none-added) concentrations of copper sulphate. RT-PCR was performed using the primers: pmo+7F (GACGAGGGTTTTCGGCG) and O2 (TGTTGTTACGCTCATCTC) for *pmo*; and 206F (ATCGCBAARGAATAYGCSCG) and 886R (ACCCANGGCTCGACYTTGA A) for *mmoX* (included to validate low-copper [*mmo*-expressing] conditions). It is clear from these results that the *pmo* operon was always transcribed under the conditions tested and the *mmo* (*mmoX*) operon only under copper-limited conditions (Table 4.1). Also, soluble methane monooxygenase (sMMO) activity was assayed qualitatively using the naphthalene oxidation assay (Brusseau *et al.*, 1990) which showed that sMMO activity was only detectable from cells grown in low copper medium.

Table 4.1 Transcription of *pmo* and *mmo*, in medium containing 0 and 5 μM added $CuSO_4$, with methane as sole carbon and energy source. Identical results were obtained from independent duplicate cultures. Positive controls using *M.trichosporium* OB3b DNA and negative controls to check DNA contamination of RNA samples were performed in all cases. A positive result (+) indicates the presence of the RT-PCR product of expected size for the appropriate gene, and negative (-) its absence.

[CuSO4] μM	*pmo* RT-PCR	*mmo* RT-PCR	Naphthalene assay
0	+	+	+
5	+	–	–

4.3 Transcription of methane monooxygenase genes during growth on methanol : effects of copper ions

M. trichosporium OB3b is also capable of growth using methanol as its sole carbon and energy source, but little is known about the expression of the two methane monooxygenase enzymes whilst the organism is using methanol. Thus, the aim of this work was to qualitatively assay for *mmo* and *pmo*-specific transcripts from RNA extraceted from cells grown on methanol in the presence of varying concentrations of copper.

Total RNA was extracted from triplicate batch cultures grown in NMS with the specified amount of added copper and either 0.05 or 0.1% methanol. Prior to their innoculation into these media the cells were adapted to methanol by growth for four days on 0.05 % methanol before innoculation of 1 ml of this culture into the specified media. RT-PCR was performed using the primers listed in section 4.2. Positive controls using DNA from *M. trichosporium* OB3b as template, PCR negatives using water and RT-PCR negatives to check for DNA contamination in RNA preparations were performed in all cases.

Table 4.2 Transcription of *pmo* and *mmo* during growth on methanol.
Identical results were obtained from independent duplicate cultures. Positive controls using *M.trichosporium* OB3b DNA and negative controls to check DNA contamination of RNA samples were performed in all cases. Methanol concentrations are indicated as percentages (v/v) added prior to innoculation. ND- Not Done.

	pmo RT-PCR		*mmo* RT-PCR		Naphthalene assay	
[$CuSO_4$] μM	0.05 %	0.1 %	0.05 %	0.1 %	0.05 %	0.1 %
0	+	+	-	-	-	-
1	+	ND	-	ND	-	ND
2	+	ND	-	ND	-	ND
5	ND	+	-	-	ND	-

A qualitative analysis of the transcription of *mmo* and *pmo* operons transcription from cells grown using methanol as sole carbon and energy source revealed that the *pmo* operon was transcribed under all conditions tested. Thus, transcription of the *pmo* operon appears to be constitutive, regardless of the carbon source used for growth.

In contrast, *mmo* specific transcripts were not detected in cells grown under any of the copper concentrations tested. This is surprising since *mmo* transcripts were detected under identical conditions with methane as the sole carbon and energy sources. These results indicate that transcription of the *mmo* operon is affected by the carbon source in addition to the concentration of copper present in the growth medium.

4.4 Transcription of the *mmo* operon : effects of methanol on *mmo* expressing cultures of *M. trichosporium* OB3b

The aim of these experiments was to determine whether the absence of *mmo* transcripts from cells grown with methanol as sole carbon and energy source was due to inhibition of transcription by methanol. *Methylosinus trichosporium* OB3b was grown in low copper NMS media to an OD_{540} of 0.3, before addition of 0.1% methanol to the cultures. The converse experiment where methane (20% (v/v)) was added to cultures which had been grown to an OD_{540} = O.3 using methanol as sole carbon and energy source, were also performed. In all cases, triplicate cultures were harvested for RNA extractions, at the time of addition and at time points up to 8 hours post methanol/methane addition. Once again the *mmoX*-specific primers 886R and 206F were used.

These experiments showed that the addition of methanol resulted in a loss of detectable naphthalene activity after 15 min, but that the *mmoX* transcripts were present even 8 h after the addition of methanol (Table 4.3, Figure 4.2). It may be that the levels of sMMO enzyme activity were below the detection levels of the naphthalene assay used here and that transcription still occurred at a low but detectable level by RT-PCR. In the converse experiments, where methane was added to methanol grown cultures, no *mmoX* transcripts were detected at any time (data not shown).

Table 4.3 RT-PCR and naphthalene assay for *M. trichosporium* OB3b grown on methane with methanol added at time = 0. Times refer to minutes after addition of methanol (0.1%) to cultures at OD_{540} = 0.3.

Time (mins)	mmo RT-PCR	Naphthalene assay
0	+	+
15	+	-
30	+	-
60	+	-
120	+	-
240	+	-
480	+	-

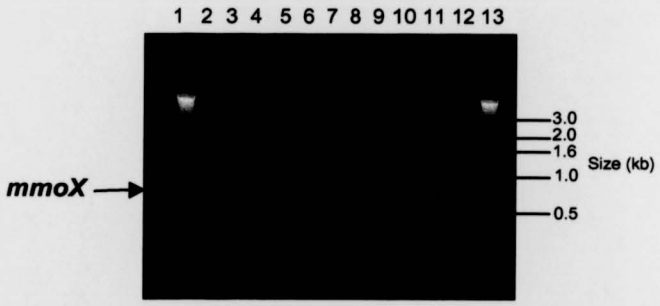


Figure 4.2 Typical *mmoX* -specific RT-PCR gel.
The primers 206F and 886R (as listed in Figure 4.1) were used for RT-PCR on RNA extracted from methane grown wild-type *M. trichosporium* OB3b cells (lanes 2-8) with methanol (0.1% v/v) added at time= 0, samples were taken at 0 h (lanes 6-8), 4 h (Lanes 2-3) and 8 h (Lanes 4-5). Negative controls containing RNA only were performed separately and shown not to contain *mmo*-specific DNA. Additional negative controls using strain Gm1 (*mmo*$^-$) of *M. trichosporium* OB3b (lanes 9-10) (Chapter 5), no DNA (Lane 12) were also performed. The positive control contained wild-type DNA (Lane 11). 1 kb DNA ladder (Gibco BRL) was used as a size marker.

In light of the data presented in this section it could be predicted that *mmoX* transcription should be detected in cells grown solely with methanol as its sole carbon and energy source. However, *mmoX* transcripts were never detected in cells grown solely with methanol as the sole carbon and energy source. This may indicate something different about cells grown solely with methanol as a sole carbon and energy source which does not allow *mmo* transcription. Alternatively, methane may be required in addition to low copper concentrations for *mmo* transcription.

4.5 Discussion

In this chapter data have been presented showing that the *pmo* operon is transcribed, to some degree, in cells grown with methane or methanol at high and low copper concentrations. This agrees with recent findings by Stolyar *et al.*, (2001) in which *pmo* specific transcripts were detected from *Methylococcus capsulatus* Bath cells grown with methane as sole carbon and energy source in the presence of high and low copper levels from. The transcription of the *pmo* operon even at low copper levels may indicate that it plays a role in the cell other than in methane oxidation under these conditions. It is well established that copper is very important to this organism and that *Methylosinus trichosporium* OB3b possesses a specialised system for copper uptake. Dispirito *et al.*, (1998) have shown that *M. trichosporium* OB3b releases copper-binding compounds (CBCs) into the growth medium under times of copper stress (see section 1.10.1). If the pMMO enzyme plays a role in acquisition of copper from the environment via the CBCs then you could predict that it would be present even at low copper concentrations. Whereas if it's sole function were in methane oxidation, its complete absence at low copper concentrations may be expected. Interestingly, the CBCs can be found in association with the pMMO enzyme (Zahn and Dispirito, 1996), suggesting that it may indeed play a role in acquisition of copper via these compounds.

In this Chapter, data are also presented which show the presence of *pmo* transcripts from cells grown with methanol as sole carbon and energy source. Studies of pMMO activity from *M. trichosporium* OB3b, by Ruth Finch (1997) using low-copper chemostat cultures with excess methane and limiting methanol concentrations revealed the presence of an inactive pool of pMMO enzyme. When pMMO activity was assayed in cells taken from these low-copper chemostats growing with 50 and 500mM methanol in the growth medium, pMMO activity increased with increasing methanol concentrations. These results indicated that in methanol-grown cells there was a pool of pMMO enzyme which increased in the presence of excess methanol. The detection of *pmo* transcripts under these conditions seems to support these findings and may represent a copper-independent activity of the pMMO complex in protecting the cell from toxic levels of methanol. These data would be complemented by a study of the levels of *pmo* transcripts in the presence of methanol. There are two copies of the *pmo* operon in *Methylosinus trichosporium* OB3b which may respond

differently to environmental stimuli, as shown for *Methylococcus capsulatus* Bath (Stolyar *et al.*, 2001). An analysis of transcription of the two copies of the *pmo* operon from *M. capsulatus* Bath revealed that copy two was predominantly transcribed below 5mM copper and that transcription of copy one was elevated to the same level at higher copper concentrations (Stolyar *et al.*, 2001). The construction of copy-specific promoter fusions would allow the levels of transcription from these promoters to be monitored in response to methanol (and copper) and may shed light on this interesting phenomenon. This could be achieved by the cloning by PCR of the regions 5' of *pmoC* for the two copies of the *pmo* operons from *M. trichosporium* OB3b into the vector pMFX1 (Stolyar *et al.*, 2001). This vector was constructed in the laboratory of Mary Lidstrom, University of Washington, USA; and contains a promoterless *xylE* reporter gene, kanamycin resistance, narrow host-range origin of repplication and the RP4-*mob* cassette. This vector allows the construction of transcriptional fusions with the reporter gene *xylE* which can be transferred into methanotrophs and inserted into the chromosome by single crossover recombination. The expression from the promoter can then be followed in cells grown on methanol and methane. What is clear form these experiments presented here is that the *pmo* operon is transcribed to some extent under all conditions tested and it is likely that it has some other function in cells grown on methanol other than methane oxidation.

Transcription of the *mmo* operon is known to occur only at low-copper concentrations (Nielsen *et al.*, 1996, 1997). The data presented in this study confirm this observation and validates the RT-PCR method used here. However, little is known regarding expression or transcription of the *mmo* operon when cells are using methanol as the sole carbon and energy source. Data presented here have shown that *mmo* transcripts remain when cells are grown on methane and methanol is added during growth. However, when cells are grown solely on methanol or have methane added during growth on methanol, *mmo* transcripts are absent. These data mirror the results of sMMO activity assays and Western blotting experiments performed by Ruth Finch (1997). sMMO activity was never detected from *M. trichosporium* OB3b cells grown with methanol only, but low levels of activity were detected when cells were grown on excess methane in the presence of methanol. Western blots also showed that there were much lower levels of sMMO hydroxylase subunits in cells grown with methane plus methanol when compared to methane only. However, Western blots performed on cell free extracts from cells grown with methanol only suggested that

not all subunits of the hydroxylase were present. In summary the data presented here in tandem with the data presented by Ruth Finch suggest that sMMO transcription and expression is partially inhibited in cells grown on methane and methanol. However, when cells are grown with methanol as the sole carbon and energy source, expression and transcription of at least *mmoX* (and thus the α-subunit), is repressed. Thus, it seems that cells grown in the presence of methanol may be subtly different from those grown with methane. It is possible that growth using methanol as a sole carbon and energy source causes the build-up of a metabolic product of methanol oxidation which in some way inhibits *mmo* transcription, a candidate is formaldehyde, the product of methanol oxidation by methanol deydrogenase.

This study focussed only on the transcription of *mmoX*. The results presented in Chapters 5 and 6 suggests that the promoter 5' of *mmoY* is functional and it may be that it responds to methanol differently to *mmoX*. Future work should focus on the transcription of *mmoX* and *mmoY* at a quantititive level using a combination of RT-PCR and Northern blotting (see section 8.2).

What has emerged from this study is that transcription of the *pmo* and *mmo* operons is not solely responsive to copper and that the carbon source may also play a role in the regulation of this complex genetic system. It is also clear that the two methane monooxygenase operons respond in different ways to methanol and that the pMMO enzyme may have other functions in these cells in addition to methane oxidation.

Chapter 5

Cloning and phenotypical characterisation of *rpoN* from *Methylosinus trichosporium* OB3b

5.1 Introduction

In contrast to the promoters identified 5' of the *pmoCAB* operon in *Methylosinus trichosporium* OB3b (described in chapter 3), the promoter identified upstream of the *mmo* operon (encoding the sMMO enzyme) possesses high similarity to a σ^N consensus promoter: TGGCA-N_6-TTGCa/t (Nielsen *et al.,* 1997). Initiation of transcription from these promoters requires the involvement of the σ^N subunit of RNA polymerase and a transcriptional activator protein (Merrick *et al.,* 1993; Buck *et al.,* 2000). The σ^N protein is encoded by *rpoN* (formerly *ntrA* and *glnF*) and is denoted *rpoN* due to the significant role it plays in nitrogen metabolism in many bacteria. In addition to its role in the transcriptional regulation of nitrogen metabolism genes, it is now known to be involved in the regulation of a wide variety of metabolic processes in many bacteria (reviewed in Merrick, 1993; Buck *et al.,* 2000; Studholme and Buck, 2001).

Possibly the most well known example of σ^N regulation is the *glnA* operon of *E.coli,* encoding the glutamine synthetase (GS) enzyme, which is responsible for the assimilation of low concentrations of ammonia in this organism (Merrick & Edwards, 1995). The *glnA* gene, is transcribed in times of ammonia limitation by the action of the σ^N protein and NtrC. NtrC is a member of a family of proteins which are always required for positive activation of gene transcription from σ^N-type promoters. However, in this case transcriptional regulation is not the only level of control, both NtrC and GS are post-translationally modified in response to ammonia levels (Merrick & Edwards, 1995).

The presence of a σ^N-type promoter located 5' of the *mmoX* gene in *M. trichosporium* OB3b indicates that σ^N may play a role in the transcriptional regulation of the soluble methane monooxygenase gene cluster in this organism. Its historical role with respect to nitrogen regulation also suggested that σ^N had a role in control of nitrogen metabolism in this organism (See section 1.9). In contrast, the promoter found upstream of *mmoX* in *Methylococcus capsulatus* Bath possesses a region identical to the –24 motif of a σ^N -type promoter, but no highly conserved –12 motif. Thus it is unclear whether the *mmo* operon in *Methylococcus capsulatus* Bath is controlled by σ^N.

In the work described in this chapter the *rpoN* gene from *Methylosinus trichosporium* OB3b was cloned, sequenced and mutated in order to examine its role in regulation of both the soluble methane monooxygenase and nitrogen metabolism. Attempts were also made to perform similar experiments with the *rpoN* from *Methylococcus capsulatus* Bath. This information would provide information on the novel copper-dependent regulation of methane monooxygenase enzymes and the nitrogen regulatory circuits in these two methanotrophs.

5.2 Cloning of the *rpoN* gene cluster from *Methylosinus trichosporium* OB3b

In order to clone the *rpoN* gene from *Methylosinus trichosporium* OB3b, a Southern blotting approach was used to identify candidate *rpoN*-containing fragments in the chromosome.

5.2.1 Cloning and sequencing using *rpoN* from *Azotobacter vinelandii* (clone 355)

Chromosomal DNA from *M. trichosporium* OB3b was first probed with the *rpoN* genes from *Azotobacter vinelandii* and *Klebsiella pneumoniae* which were amplified by PCR from the plasmids pAT705 and pMM17, respectively (Merrick and Stewart, 1985; Merrick and Gibbins, 1985). The *rpoN* from *Azotobacter vinelandii* was amplified using the primers: S54VINEF (ATGAACCATCGCTAGTCCT) and S54VINER (TTACATAAGCCGCTTGCGTT); and from *Klebsiella pneumoniae* using the primers: S54KLEBF (CACACCAGCATCGCCTTGTC) and S54KLEBR (CTCATATCCGCTGTACC). These probes were used to probe Southern blots of *Sal*I, *Pst*I, *Eco*RI or *Bgl*II digested *Methylosinus trichosporium* OB3b chromosomal DNA. The pMM17 (*K. pneumoniae*) derived probe failed to highlight any hybridising fragments, even at low stringency (2 x SSC, 20°C) whereas the pAT705 (*A. vinelandii*) derived probe allowed the identification of a 2.1 kb *Eco*R1 fragment as a putative *rpoN*-containing fragment (Figure 5.1).

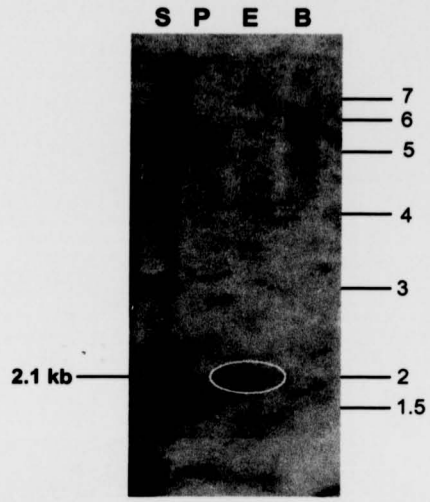


Figure 5.1 Southern blot of *Methylosinus trichosporium* OB3b chromosomal DNA probed with *rpoN* gene from *Azotobacter vinelandii*.. The *Azotobacter vinelandii rpoN* gene was amplified from pAT705 using the primers S54VINEF (ATGAACCATCGCTAGTCCT) and S54VINER (TTACTATAAGCCGCTTGCGTT) before hybridisation overnight at 65°C followed by washing in 2 x SSC at 20°C before exposure to X-ray film for 48 hours at -80°C. Chromosomal DNA from *M. trichosporium* OB3b was digested with S= *Sal*I, P=*Pst*I, E=*Eco*RI, B=*Bgl*II. The 2.1 kb target *Sal*I fragment is circled.

A partial genomic library of *Eco*R1 digested chromosomal DNA from *M. trichosporium* OB3b in the size range 1.5-3 kb was produced using the vector pUC19 and colony blotting was used to screen 600 clones containing DNA inserts. The 20 clones that hybridised most strongly to the *Azotabacter vinelandii rpoN* probe were chosen for further investigation. Plasmid preparations were performed and the plasmid DNA digested with *Eco*R1 and fragments resolved on an agarose gel, before Southern transfer to a Nylon membrane. Of these 20 clones, none were highlighted when challenged with the same probe (*rpoN* from pAT705). It was suspected that this observation was caused by non-specific hybridisation between the derived probe and the chromosomal copy of *rpoN* from *E.coli* TOP10 strain. In order to overcome this problem, a pooled miniprep method was employed whereby clones were inoculated into 10 ml of LB broth in groups of ten and the total plasmid DNA extracted by alkaline lysis. These plasmid DNA preparations were digested with *Eco*R1, run on agarose gels, Southern blotted and challenged with the pAT705 (*A. vinelandii*) derived *rpoN* probe. In this way, 440 clones were examined in 44 pools. One of these pools, pool 36 (clones 351-360), contained a DNA fragment of 2.1 kb which hybridised to the *rpoN* probe (data not shown). Plasmid DNA was extracted

each of the individual clones within pool 36. Southern hybridisation analysis identified that clone 355 contained the 2.1 kb *Eco*R1 fragment of *M. trichosporium* OB3b which hybridised to the *rpoN* probe.

Partial sequencing of clone 355 revealed a probable pyrroquinoline quinone (*pqq*) biosynthesis cluster. DNA sequence from clone 355 contained significant identity to the *pqqB(C/D)E* genes from the *Methylobacterium extorquens* AM1 *pqq* cluster (established using the BlastX program at http://www.ncbi.nlm.nih.gov/Blast) (Figure 5.2). The DNA sequence quality obtained from clone 355 was poor and it was not possible to produce a continuous sequence or establish the start and stop points for these open reading frames. However, it was clear that the *rpoN* gene was not contained in clone 355. Pyrroquinoline quinone (PQQ) is the prosthethic group for several bacterial enzymes, including the methanol dehydrogenase of *Methylobacterium extorquens* and other methylotrophs (Toyama *et al.*, 1997). Although it is unclear why this cluster was highlighted by these experiments, its cloning may prove useful for the future study of enzyme cofactors in *Methylosinus trichosporium* OB3b.

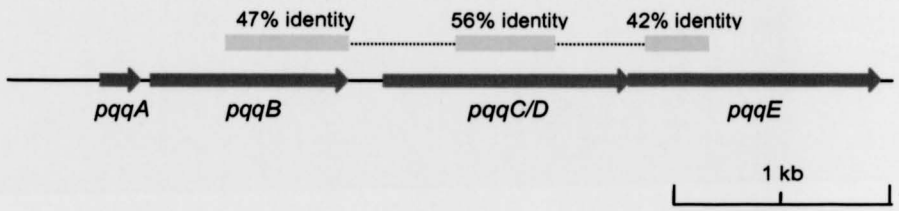


Figure 5.2 Schematic representation of sequenced portions of p355 aligned with the *pqq* cluster from *Methylobacterium extorquens* AM1. Amino acid identities are shown above the sequenced portions of clone p355 (denoted by orange boxes). Genetic organisation and nomenclature follows scheme proposed by Toyama *et al.*, (1997).

5.2.2 Chromosomal probing using *rpoN* from *Methylococcus capsulatus* Bath

A similar approach to that described in section 5.2.1 was followed using the *rpoN* gene from the methanotroph *Methylococcus capsulatus* Bath. The sequence of this gene was a kind gift from Dr Oivind Larsen, University of Bergen, Norway; a member of the genome sequencing project for this organism (TIGR and the University of Bergen, Norway). A PCR-derived probe containing the whole of the *rpoN* gene was amplified using the primers: RPOMCF (AAAACTGCACATGAAACAATCACGCAAC) and RPOMCR (AAAACTGCAGTTAGAACAACTGCTTTCTTTCG) and used to probe Southern blots of *Methylosinus trichosporium* OB3b chromosomal DNA, but failed to highlight any hybridising fragments (data not shown).

5.2.3 Cloning of the *rpoN* gene from *M. trichosporium* OB3b using the *Sinorhizobium meliloti* 1021 *rpoN* gene as a probe

Recent phylogenetic studies of known *rpoN* genes revealed grouping which roughly corresponed to 16s rRNA phylogeny (Studholme and Buck 2000; Powell *et al.*, 1995). This may explain why attempts to use *Azotobacter vinelandii, Klebsiella pneumoniae* and *Methylococcus capsulatus* Bath derived probes all failed to identify an *rpoN* containing chromosomal fragment. These three organisms are members of the γ-subclass of the *Proteobacteria*, but *Methylosinus trichosporium* OB3b is a member of the α-subclass of the *Proteobacteria*. Therefore, a *Sinorhizobium meliloti* 1021 (α-subclass of the *Proteobacteria*) derived probe was used since the gene from this organism should be more closely related to the *rpoN* gene from *M. trichosporium* OB3b. The *rpoN* gene from *Sinorhizobium meliloti* 1021 was amplified by PCR, using primers S54MELF (CACACCAGCATCGCCTTGTC) and S54MELR (CTCATATCCGCTGTACC) designed from the sequence of *rpoN* published by Ronson *et al.*, (1987). This 1.5 kb PCR fragment containing the whole *rpoN* gene was then cloned into pCRTOPO-3.1 (creating pSMEL5) and sequenced to verify its identity .

Using the approach detailed in section 5.2.1, this probe identified two putative *rpoN* containing DNA fragments, a 3.7 kb *Sst*I and 3.0 kb *Sal*I fragment from the chromosome of *M. trichosporium* OB3b (Fig. 5.3). Partial genomic libraries of *Sst*I (3.0 – 5.0 kb) and *Sal*I (2.5 – 4.0 kb) digested *M. trichosporium* OB3b DNA were prepared in pUC18 vector. Due to the presence of an *rpoN* gene in the chromosome

of *Escherichia coli,* the pooled-miniprep method was again employed, screening 600 clones in groups of 10. This method identified clone 264 which contained the 3 kb *Sal*I fragment, but DNA sequencing subsequently showed that it contained only a 519 bp portion of the *rpoN* gene (data not shown). In order to clone the larger *Sst*I fragment an *rpoN* probe of 500 bp was generated by PCR from chromosomal DNA of *M. trichosporium* OB3b using primers designed from the sequence of clone 264: OB3brpoF (AGTCGACGACGAGGACATCG) and OB3brpoR (AAGAAGAAATATTTCAGCTCG). Using this homologous *rpoN* probe, clone 519 (named pGPS519) was identified from a library of 600, which contained the 3.7kb *Sst*I fragment believed to harbour the *rpoN* gene (Figure 5.4).

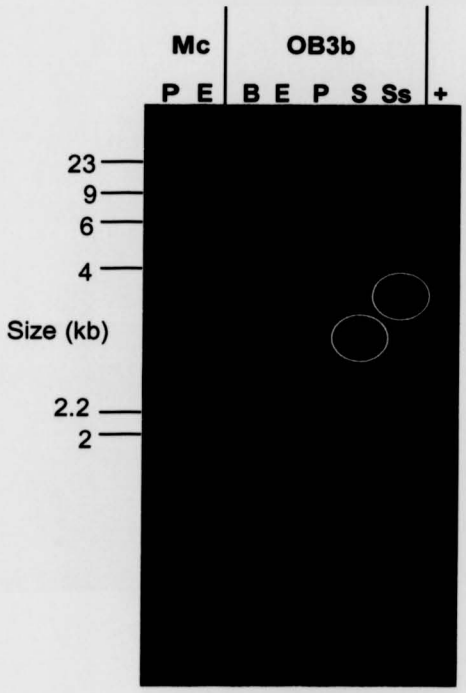


Figure 5.3 Southern blot: *Methylococcus capsulatus* Bath and *M. trichosporium*OB3b probed with the *rpoN* gene from *Sinorhizobium meliloti* 1021. Chromosomal DNA from *M. trichosporium* OB3b (OB3b) and *Methylococcus capsulatus* Bath (Mc) was digested with *Bam*HI (B), *Pst*I(P), *Sal*I (S) of *Sst*I (Ss) as indicated. Plasmid pSMEL5 digested with *Eco*RI was included as positive control. The 3.7 kb *Sst*I and 3.0 kb *Sal*I Fragments highlighted by probing with the entire *rpoN* gene from *Sinorhizobium meliloti* are circled in yellow. Washing was performed at 60°C, 2 x SSC, this autorad was exposed for 48h at –80 °C.

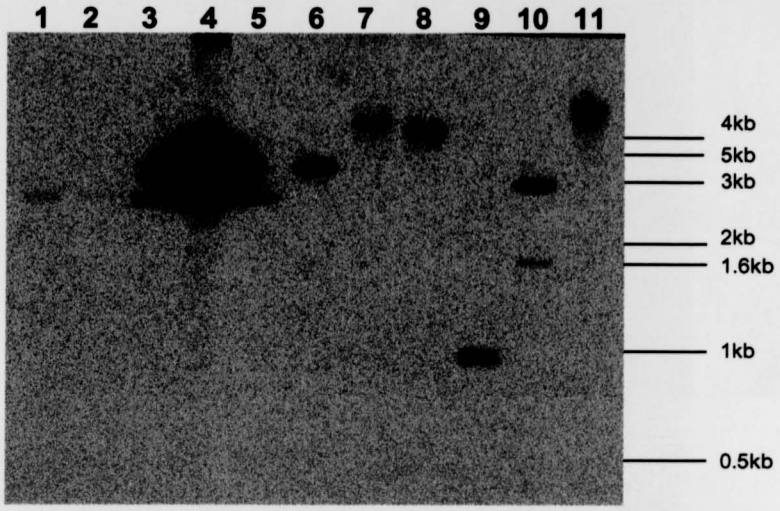


Figure 5.4 Southern blot showing the identification of clone 519 and chromosomal blot of *M. trichosporium* OB3b. This blot was probed with a PCR generated *rpoN* specific probe amplified using primers OB3brpoF (AGTCGACGACGAGGACATCG) and OB3brpoR (AAGAAGAAATATTTCAGCTCG) from the chromosome of *M.trichosporium* OB3b. Washing was performed at 60°C, 1xSSC. Lanes 1-5 represent individual clones digested with *Sst*I: 1, Clone 516; 2, Clone 517; 3, Clone 518; 4, Clone 519; 5, Clone 520; Lanes 6-11 represent chromosomal DNA of *Methylosinus trichosporium* OB3b digested with: 6, *Sst*I; 7, *Bam*HI; 8, *Bgl*II; 9, *Pvu*II; 10, *Sal*I; 11, *Xba*I.

5.2.4 DNA sequence analysis of the *rpoN* cluster from *Methylosinus trichosporium* OB3b

The nucleotide sequence of the *Sst*I fragment from pGPS519 was determined. It contained 1272 bp of the *rpoN* gene and four other open reading frames (Figure 5.5). This clone is therefore missing approximately 250 bp from the predicted 5' end of the *rpoN* gene.

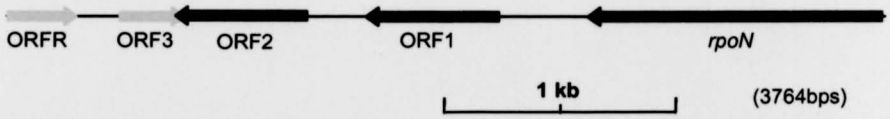


Figure 5.5 Genetic organisation of tbe sequenced region of the *rpoN* cluster from *Methylosinus trichosporium* OB3b. This sequence originates from pGPS519: pUC18 containing a 3.76 kb *Sst*I insert. Open reading frames were assigned using the ORF Finder tool in conjunction with the BlastX program (http://www.ncbi.nlm.nih.gov).

The derived amino acid sequence of the cloned portion of the *rpoN* gene from *Methylosinus trichosporium* OB3b is most closely related (~55% amino acid identity) to the RpoN of several members of the family *Rhizobiaceae* (Identified using the BlastX tool). It is a typical *rpoN* gene and contains the highly conserved *rpoN*-box motif (ARRVATKYRE) (Merrick, 1993) except that the final glutamate is replaced by an aspartate in *M. trichosporium* OB3b. A phylogenetic distance tree compiled from an alignment of the deduced amino-acid sequences of 35 *rpoN* genes revealed that the RpoN from *M. trichosporium* OB3b groups with other members of the α-subclass of the *Proteobacteria*, loosely following 16S rRNA phylogeny (Figure 5.6). The alignment was based on 307 amino acids, which excluded the hypervariable region II (Merrick, 1993; Buck *et al.*, 2000)(See Appendix B for full alignment). The deduced amino-acid sequence of the putative *rpoN* gene from *Methylococcus capsulatus* Bath was also included in this analysis and falls within a group formed by the γ-subclass of the *Proteobacteria*. The position of RpoN from *Sinorhizobium meliloti* on a branch with *Methylosinus trichosporium* OB3b confirmed the observation that its *rpoN* gene was a much better probe than either *Methylococcus capsulatus* Bath or *Klebsiella pneumoniae* in the cloning experiment.

Immediately 3' of the *Methylosinus trichosporium* OB3b *rpoN* gene lie two ORFs (Figure 5.5). The first of these, ORF1, appears to be a new member of a group of genes found immediately downstream (3') of *rpoN* genes in many other bacteria (Ronson *et al.*, 1987; Michiels *et al.*, 1998a; Warrelmann *et al.*, 1992; Merrick, 1993). It encodes a protein of 192 amino acids, and is 48% identical to the derived amino acid sequence from the corresponding gene (ORF203) from *Bradyrhizobium japonicum* (Kullik *et al.*, 1991). The only other proteins with homology to ORF1 are a spinach ribosomal protein gene (Merrick 1993) and the sequence derived from a gene (ORF113) upstream of the *pheA* gene from *E.coli* (Powell *et al.*, 1998). A putative Shine-Dalgarno sequence with high similarity to the *E. coli* consensus (AGGAGG) was located 7 bp from the start codon of ORF1: AGGTGG (Shine and Dalgarno, 1974). In addition, a DNA sequence (CGATCGAACG-N4-CGTTCGATCG) with the potential to form a stable stem-loop structure (ΔG= -10.9 kcal) was found 10 bp downstream (3') from the stop codon of *rpoN*, which may function as a transcriptional terminator.

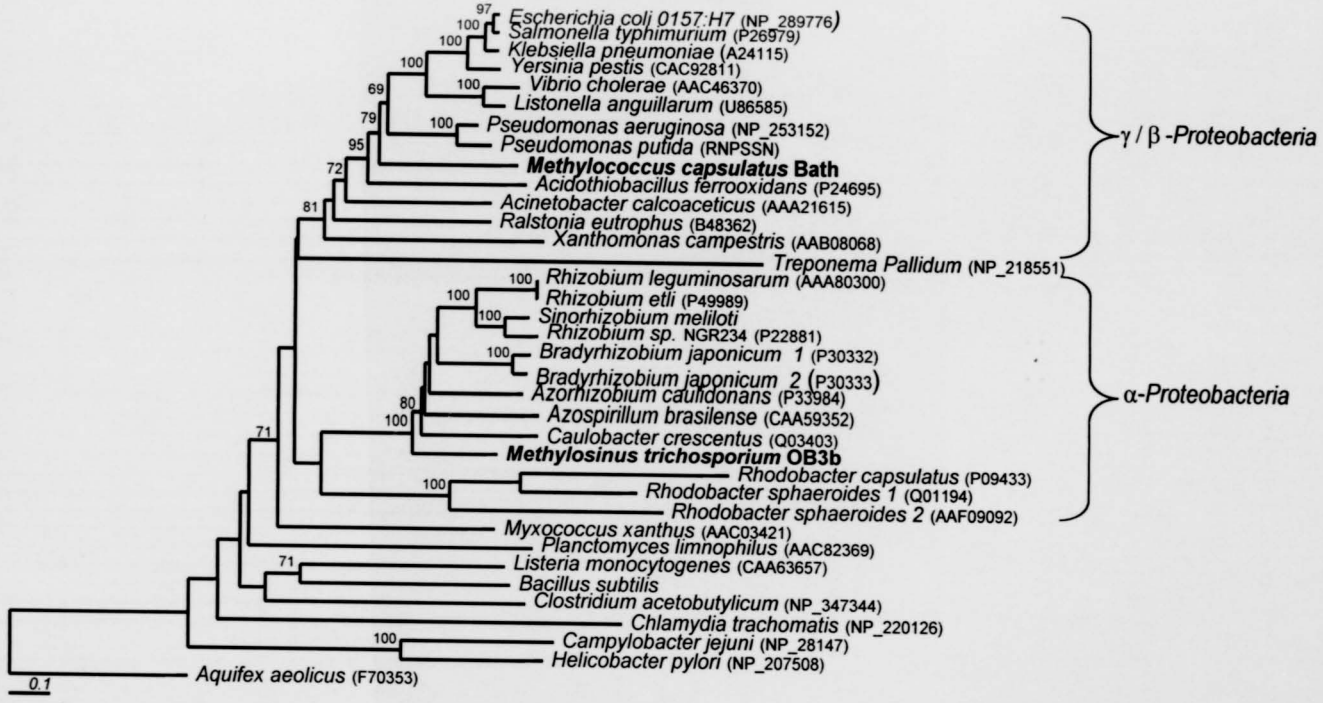


Figure 5.6 Phylogenetic distance tree of σN. Amino acid sequences were obtained from the Entrez protein database (accession numbers in brackets) The alignment was based on 307 amino acids from positions 171 to 616 (shown in appendix B). Analyses used programmes available within PHYLIP (Felsenstein, 1993). Pairwise distances were calculated with PROTDIST (Dayhoff PAM matrix) and a tree generated with FITCH. The RpoN of *Aquifex aeolicus* was chosen as the outgroup as it is the only archael RpoN available. Bootstrap analysis (SEQBOOT; 100 trees) was used to determine the reliability of branchpoints. Only values above 70% from the consensus tree (CONSENSE) are shown, as these are considered to support branch points (Zharkikh and Li, 1992)

The second Open Reading frame, ORF2, encodes a protein of 186 amino acids with a high degree of identity (64% at the amino acid level) with the gene encoding the phosphotransferase system enzyme II from *Mesorhizobium loti* (*ptsN*). A putative Shine-Dalgarno (ATGGGA) is located 10 bp 5' of the ATG codon of this open-reading frame. It is possible that this gene is co-transcribed with ORF1 as no potential stem-loop structures are present between ORF1 and ORF2.

The third Open Reading Frame (ORF3) is transcribed in the opposite direction and encodes a protein of 92 amino acids. It possesses significant identity to conserved hypothetical proteins of unknown function found in the genome sequences from organisms such as *Caulobacter crescentus, Pseudomonas aeruginosa* and *Salmonella enterocolitica* (Identified using the BlastX tool at http://ncbi.nlm.nih.gov/blast). Finally, the partial open reading frame, ORFR, of 99 amino acids has high homology (69% identity) with a putative two-component regulator gene identified from the genome sequence of *Mesorhizobium loti*.

Of the four ORFs associated with the *rpoN* gene from *Methylosinus trichosporium* OB3b, ORFs 1 and 2 are almost always found in *rpoN* operons (Merrick, 1993). The exceptions are those organisms which contain two *rpoN* genes, such as *Rhizobium etli* (Michiels *et al.*,1998b) and *Rhodobacter sphaeroides* (Meijer and Tabita, 1992) where the second *rpoN* copy lies adjacent to nitrogen fixation genes.

Southern analysis using the *rpoN* gene from *M. trichosporium* OB3b as a homologous probe revealed that there is probably only one copy of the *rpoN* gene in *Methylosinus trichosporium* OB3b (Fig. 5.4). The clear phenotypes observed in the *rpoN* mutation experiments presented in this chapter also support this suggestion.

5.3 Phenotypic characterisation of *rpoN* mutants

5.3.1 Construction of vectors for the insertional inactivation of the *rpoN* gene from OB3b

To examine the role of σ^N in the regulation of the sMMO and nitrogen metabolism genes of *Methylosinus trichosporium* OB3b, a narrow-host range, mobilisable, knockout plasmid was constructed. The plasmid used was based on a version of pBR329, which contained the RP4*mob* determinant, allowing transfer from *E. coli* S17-1 to the donor organism by conjugation whilst being incapable of maintenance in methanotrophs. The construction of a knockout plasmid involved 3 steps (illustrated in figure 5.7). Firstly, a 563 bp *Nco*I-*Hin*dIII fragment was deleted from the chloramphenicol resistance gene of plasmid pBR329*mob*. Into this modified version of pBR329*mob,* a 2.3 kb *Nco*I-*Hin*dIII fragment from pGPS519, containing *rpoN* and ORF1, was ligated. The resultant chloramphenicol-sensitive clones were analysed by restriction mapping to check for the presence of the insert from pGPS519. This plasmid was named pGPS103. The *rpoN* gene was then disrupted by the insertion of the gentamycin resistance cassette (Gm^R) from p34S-Gm into a *Bgl*II site which lies at its centre. This was achieved by the digestion of pGPS103 with *Bgl*II, which cuts only within *rpoN*, followed by the ligation of an 863 bp *Bam*HI fragment containing Gm^R, into this site to create pGPS103Gm. This plasmid (pGPS103Gm) (Figure 5.7) therefore contained a mutated version of the *rpoN* gene on a narrow-host range mobilisable vector which could then be transferred into *E. coli* S17-1 before mating with *Methylosinus trichosporium* OB3b.

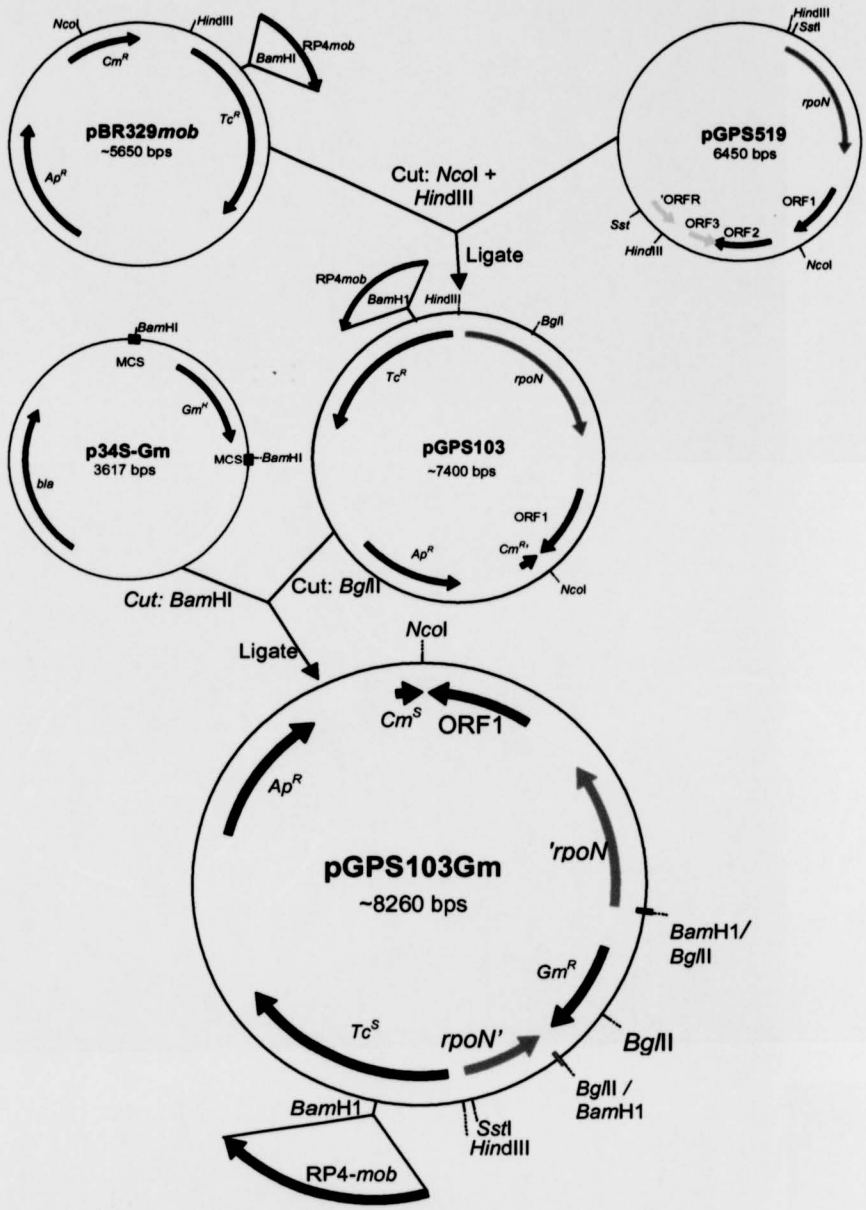


Figure 5. 7 Schematic representation of pGPS103Gm construction
Firstly, a 563 bp *NcoI-HindIII* fragment was deleted from the chloramphenicol resistance gene of plasmid pBR329*mob*. Into this, a 2.3 kb *Nco*I-*Hin*dIII fragment from pGPS519, containing *rpoN* and ORF1, was ligated to give pGPS103. The *rpoN* gene was then disrupted by the insertion of the gentamycin resistance cassette (Gm^R) from p34S-Gm into a *Bgl*II site which lies at its centre. This was achieved by the digestion of pGPS103 with *Bgl*II, followed by the ligation of an 863 bp *Bam*HI fragment containing Gm^R, into this site to create pGPS103Gm. The orientation was confirmed by restriction mapping with *Bgl*II and *Nco*I.

5.3.2 Marker-exchange mutagenesis of the *rpoN* gene of *Methylosinus trichosporium* OB3b

In order to inactivate the *rpoN* gene, a double crossover homologous recombination event between pGPS103Gm and the chromosomally encoded wild-type version of the *rpoN* gene in the chromosome must occur. This would result in the Gm^R cassette residing within the chromosomal copy of *rpoN*. This method of gene inactivation has previously been reported by Martin and Murrell (1995), who created an insertional mutant in the *mmoX* gene of *Methylosinus trichosporium* OB3b (See section 1.6). A single crossover event would result in both a wild-type (functional) and mutated version of the gene being present in the chromosome.

The knockout plasmid pGPS103Gm was transferred into *Methylosinus trichosporium* OB3b by filter mating and exconjugants were selected on NMS agar plates containing 5 μg/ml gentamycin. This method only yielded one colony from 50 ml of mating mixture, at a frequency of approximately 1×10^{-9} exconjugants per recipient cell in the original mating. However, when this strain was streaked onto NMS agar it grew very poorly. It was later discovered that the inability to use nitrate as a nitrogen source is a phenotype of an *rpoN* mutant. Therefore, the fortuitous acquisition of this mutant after growth on NMS plates was probably due to alternative nitrogen sources being released from the dead or dying bacteria on these plates. Curiously, repetitions of this experiment with selection using glutamine as a nitrogen source did not yield any exconjugants. This was probably due to the inefficiency of the transfer and double-crossover event process in this organism.

The resultant strain (Gm1) was gentamycin resistant and ampicillin sensitive, indicating the loss of the plasmid from the cell, and thus a likely double-crossover event. PCR analysis of DNA from the Gm1 strain and a putative single recombinant strain Gm2 generated in a parallel experiment (section 5.4.2), revealed that the Gm1 strain contained only a mutant version of *rpoN*, whereas the Gm2 strain contained both mutant (1588 bp) and wild-type (723 bp) PCR products (Fig 5.8). This pattern of PCR products indicated that a double crossover had occurred between the plasmid pGPS103Gm and the chromosomal copy of *rpoN*.

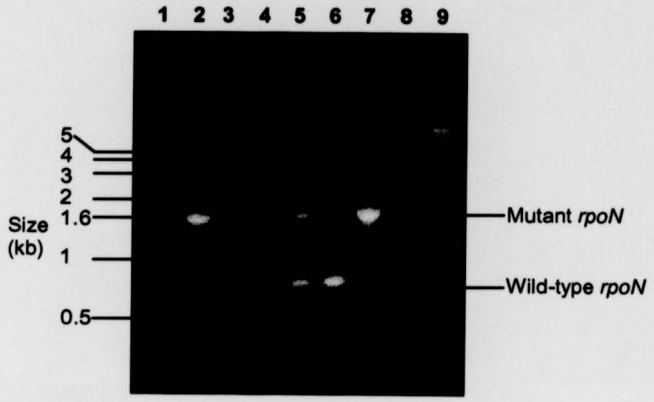


Figure 5.8 *rpoN* specific PCR of Gm1 and Gm2 mutants of *Methylosinus trichosporium* OB3b. *rpoN*-specific PCR product was amplified using primers rpoF6 (TTGACGCGGTCGGCTATC) and OB3brpoR (GAGAAGAAATATTTCAGCTC). The wild-type PCR product is 723 bp and the mutant form 1588 bps. Lanes: 1, 1kb ladder; 2, Gm1 mutant chromosomal DNA; 3, Gm1 mutant colony PCR; 4, Blank; 5, Gm2 Mutant Chromosomal DNA; 6, Wild-type chromosomal DNA; 7, pGPS104 DNA; 8, negative control; 9, 1 kb ladder.

Southern analysis of strain Gm1 also indicated the presence of a mutant copy of the *rpoN* gene, although incomplete digestion of Gm1 chromosomal DNA made interpretation complex (Figure 5.9A). An *Eco*R1 digest of *M. trichosporium* OB3b wild-type DNA probed with the *rpoN*-specific PCR product gave one hybridising band at approximately 2.6 kb which should increase by 865 bp (the size of the Gm^R cassette) in a mutant strain. Therefore a double crossover should contain a band at 3.5 kb. In the case of a single crossover mutant there are two possibilities: 1) [3.5 + 6.7] or 2) [2.6 (wt copy) + 7.5kb] (Figure 5.9C). However, independent triplicate Southern blots showed that the Gm1 mutant contained a band at 3.5 kb (presumably the mutant *rpoN*'Gm^R copy) and another larger band at approximately 8 kb (Figure 5.9B). In one case, this larger band was absent and a smaller 2.1 kb fragment was present (Figure 5.16, lane 13). Probing with the Gm^R cassette produced similar results.

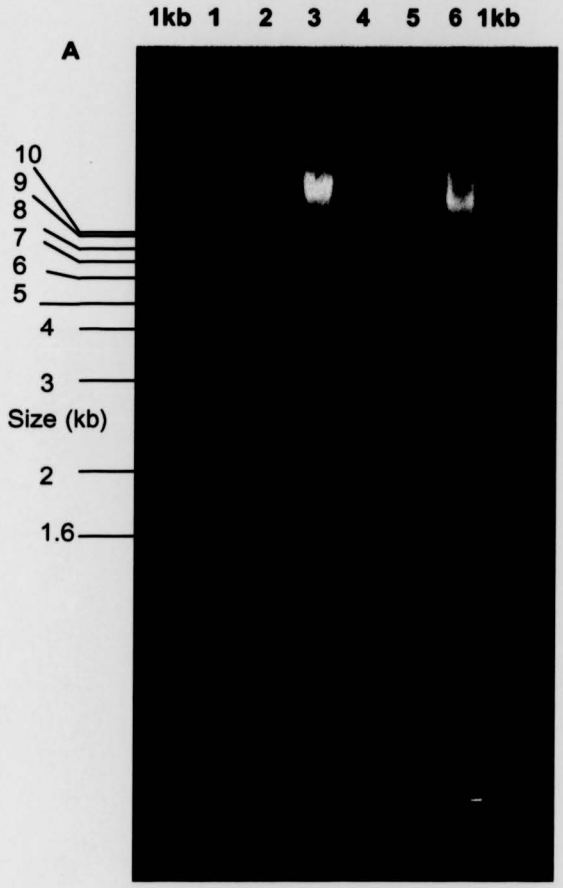


Figure 5.9A Restriction digest of chromosomal DNA from *M. trichosporium* OB3b WT (1-3) and Gm1 (4-6) strains.
Samples were resolved using a 0.8% TBE Agarose. Chromosomal DNA from WT and Gm1 strains of *M. trichosporium* OB3b were digested with the following : 1, WT *Eco*R1; 2, WT *Sst*I; 3, WT *Xba*I; 4, Gm1 *Eco*RI; 5, Gm1 *Sst*I; 6, Gm1 *Xba*I.

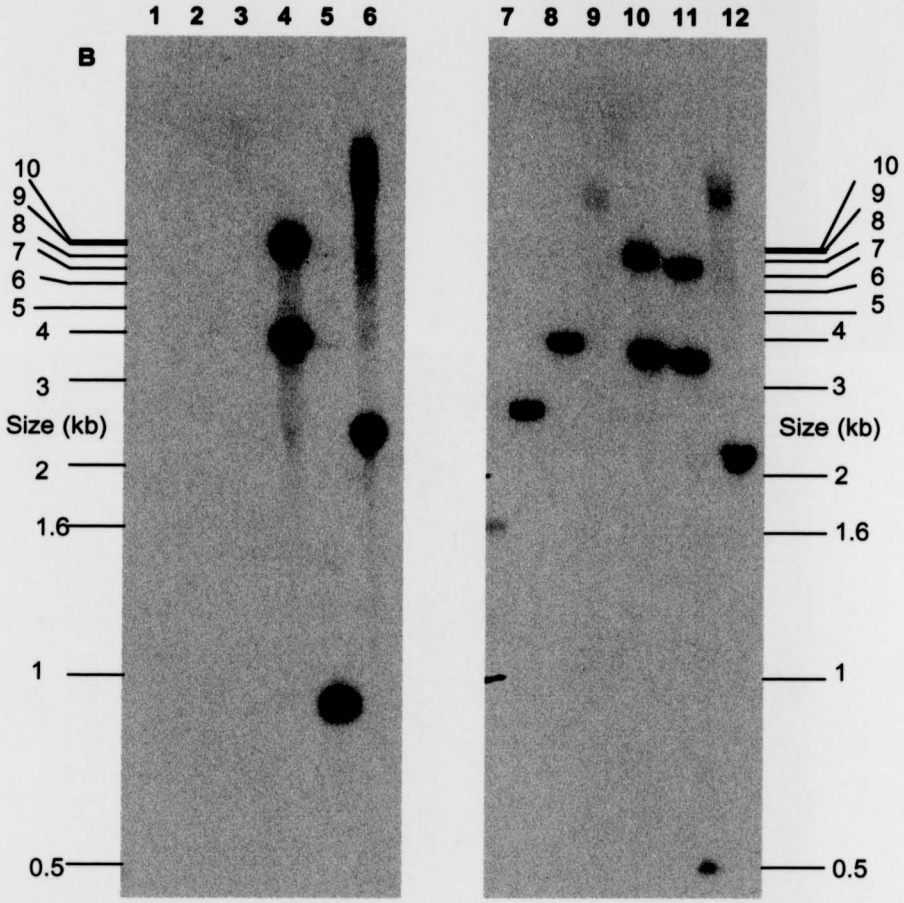


Figure 5.9B Southern blot of chromosomal DNA from *Methylosinus trichosporium* OB3b strain WT andGm1probed with Gm^R (1-6) and *rpoN* from *M. trichosporium* OB3b.
Lanes 1-6 have been probed with Gm^R cassette from p34S-Gm and lanes 7-12 by *rpoN*-specific PCR product generated by primers rpoF6 and OB3rpoR. DNA was digested with the following enzymes prior to blotting: 1+7, WT *Eco*R1; 2+8, WT *Sst*I; 3+9, WT *Xba*I; 4+10, Gm1 *Eco*R1; 5+11, Gm1 *Sst*1; 6+12, Gm1 *Xba*I. Washes were performed using 0.5xSSC at 65°C.

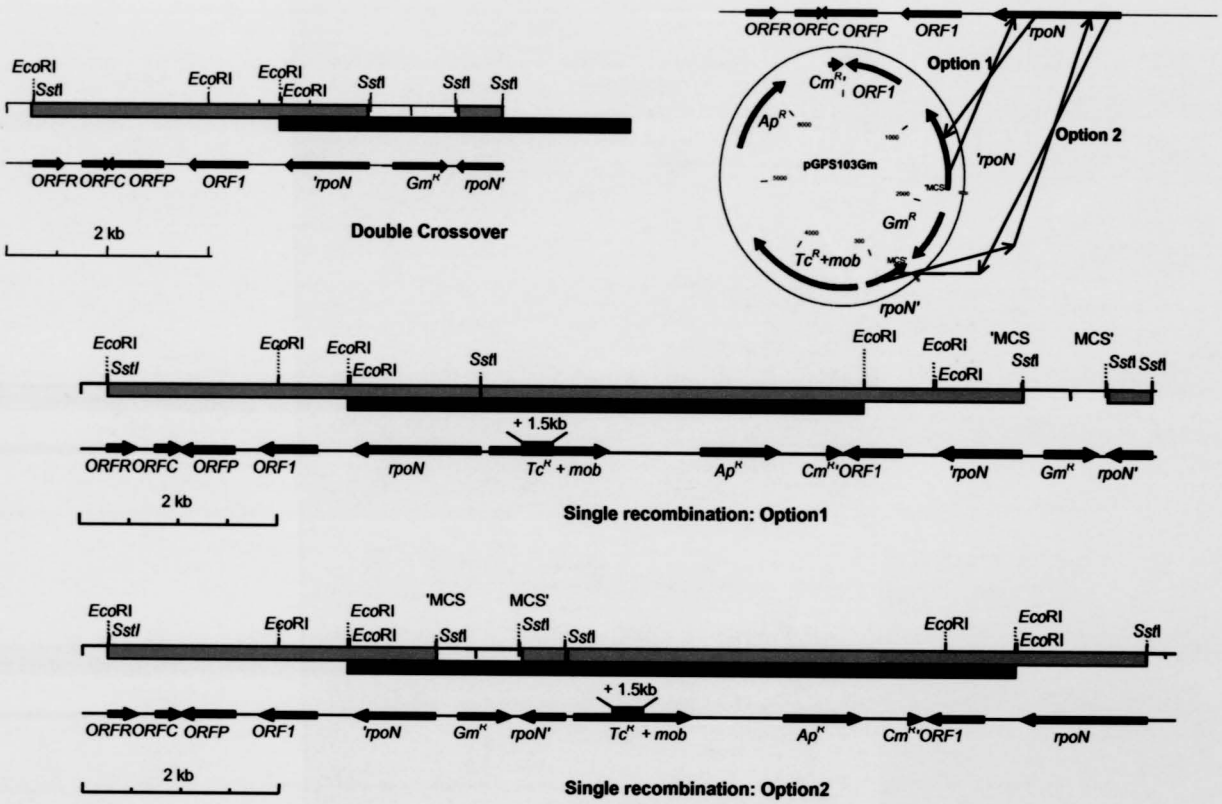


Figure 5.9C Genetic organisation of double and single recombination events after homologous recombination between the *rpoN* gene from pGPS103Gm and the chromosome of *Methylosinus trichosporium* OB3b. Fragments expected to hybridise to OB3b *rpoN* probe are highlighted in red (*Sst*I) and Blue (*Eco*R1)

A consideration of the *Sst*I digests is also confusing. In this case the wild-type band is 3.7 kb. The Gm^R cassette is flanked by *Sst*I sites which would result in a 3.2 kb (containing the 3' end of *rpoN* and the rest of the cluster) and a 0.5 kb fragment (the 5' end of the *rpoN* gene) if a double recombinant was probed with the *rpoN*-specific probe (Figure 5.9C). A single recombinant would contain: 3.7 kb (Wild-type *Sst1 rpoN* fragment), a 0.5 kb and 8.0 kb piece; OR a 3.2 kb, 10.6 kb and 0.5 kb fragments (figure 5.9C). The actual blots show 3.2 kb, 0.5 kb and 7.4 kb fragments(Figure 5.9B). Once again not totally supporting either a single or double crossover event. When probed with the Gm^R cassette, only the cassette (863 bp) is detected due to its excision by *Sst*I.

These blots do not definitively confirm the Gm1 mutant as a double-crossover mutant but neither do they reject it either. Several observations seem to favour the double-crossover event:

1: If a single crossover had occurred, then, in one orientation, a wild-type *Sst*I band would be seen in Southern blots and in the other orientation, a wild-type *Eco*RI fragment. There are no wild-type *Eco*RI or *Sst*I fragments in the Gm1 mutant blots when probed with *rpoN*.

2: A single crossover event would contain a copy of the plasmid backbone in the chromosome and therefore an ampicillin resistance gene. The Gm1 strain is ampicillin sensitive.

3: Inspection of the gels from which the blots were prepared indicate that the DNA of the Gm1 mutant was not as well digested as the wild-type DNA. Repeated attempts to overcome this problem failed. Therefore, the larger bands could originate from incomplete digestion of the DNA.

4: PCR analysis of the Gm1 mutant reveals that a wild-type copy of *rpoN* no longer resides in its chromosome. In contrast, a single crossover mutant generated in a parallel experiment contained both wild-type and mutant PCR products.

Most importantly, the Gm1 mutant has several clear phenotypes indicating that regardless of whether this mutant is a single or double crossover, it does have a disrupted *rpoN* gene.

Thus, these data indicate that the *rpoN* gene of *Methylosinus trichosporium* OB3b has been interrupted by the insertion of a Gm^R cassette using marker-exchange mutagenesis.

5.3.3 Effects of *rpoN* knockout on sMMO expression

To establish whether the *rpoN* gene product (σ^N) had a role in regulation of the *mmo* operon, it was necessary to test the effects of this knockout on the expression of the sMMO at three levels: activity, transcription and protein translation. In addition, the effects on the ability of such a mutant to grow under low copper conditions was tested.

The activity of the sMMO enzyme was tested qualitatively using the naphthalene assay (Brusseau *et al.*, 1990). The Gm1 and Wild-type strains were grown for 10 days on MS-Glutamine (0.05% w/v) low copper plates and assayed for naphthalene oxidation. Colonies expressing sMMO (wild-type) appeared purple on the addition of tetrazotized-*o*-dianisidine, whereas those not expressing sMMO (and thus pMMO) remained orange. It is clear from both the plate and liquid batch culture assay that the Gm1 (*rpoN* $^-$) strain does not possess a functional sMMO enzyme (Figure 5.10). However, this assay only indicates the absence of sMMO activity in the Gm1 strain and the same results could have originated from a non-functional sMMO enzyme which is still transcribed and translated.

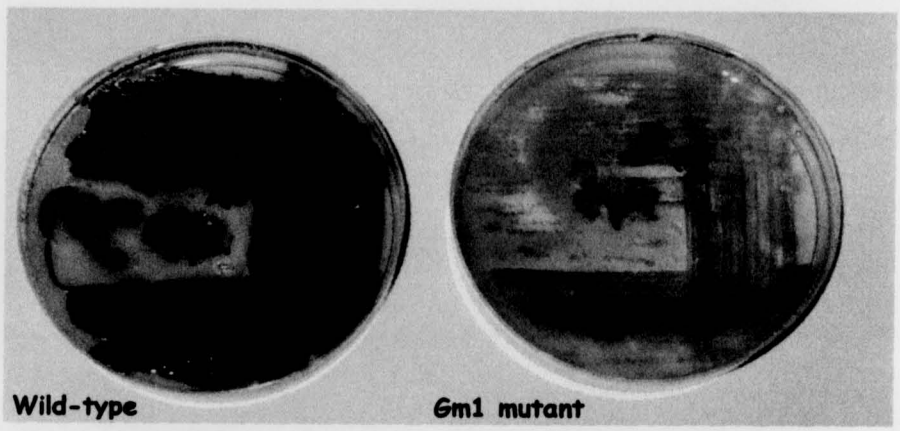


Figure 5.10 sMMO activity of Wild-type and Gm1 strains of *Methylosinus trichosporium* OB3b. *M. trichosporium* OB3b strains WT and Gm1 were grown for 10 days on MS-Glutamine Noble agar plates containing no-added copper. sMMO activity was tested using the naphthalene assay developed by Brusseau *et al.*, (1990). After incubation of plates with naphthalene (30 min, 30°C), oxidation to naphthol was tested by flooding the plates with tetrazotized *o*-dianisidine, which turns purple in the presence of naphthol, and stays orange in its absence.

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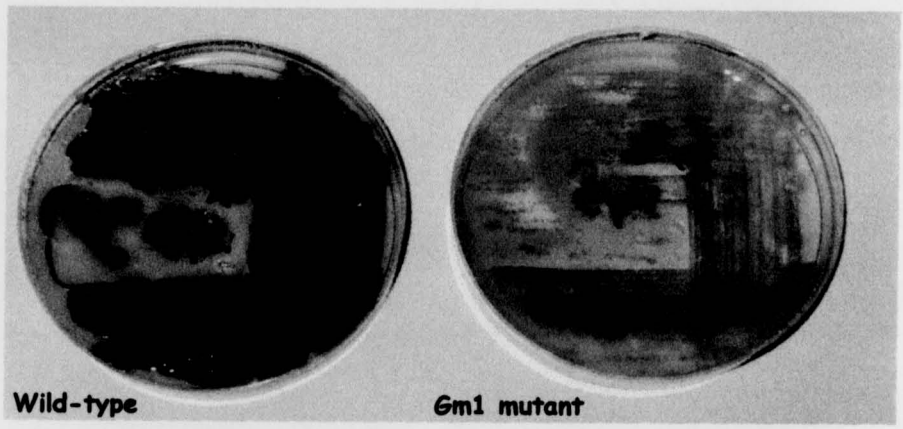

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Under low-copper growth conditions, wild-type *M. trichosporium* OB3b expresses the sMMO enzyme (Stanley *et al.*, 1983; Nielsen *et al.*, 1997). The 3 subunits of the hydroxylase component of this enzyme are clearly visible on Coomassie stained SDS-PAGE gels as polypeptides at 60, 45 and 20 kDa (α, β and γ subunits respectively). Crude cell-free extracts were prepared from 50 ml cultures of wild-type and Gm1 strains (OD_{540} = 0.5-0.6) of *M. trichosporium* OB3b grown in low-copper MS-Glutamine (0.05% w/v) medium. Approximately 90 μg of these protein extracts was run on 12% SDS-PAGE gels (Figure 5.11). The three sMMO hydroxylase subunits are clearly visible in the wild-type extracts, but are absent from the Gm1 strain. Western blots of these gels were also prepared and probed with antisera to sMMO-hydroxylase. Although there were problems with non-specific binding of the antisera to other proteins, including methanol dehydrogenase, it is clear that the α and γ subunits, but not the β were absent in cell-free extracts prepared from Gm1 cells from three independent cultures (Figure 5.11).

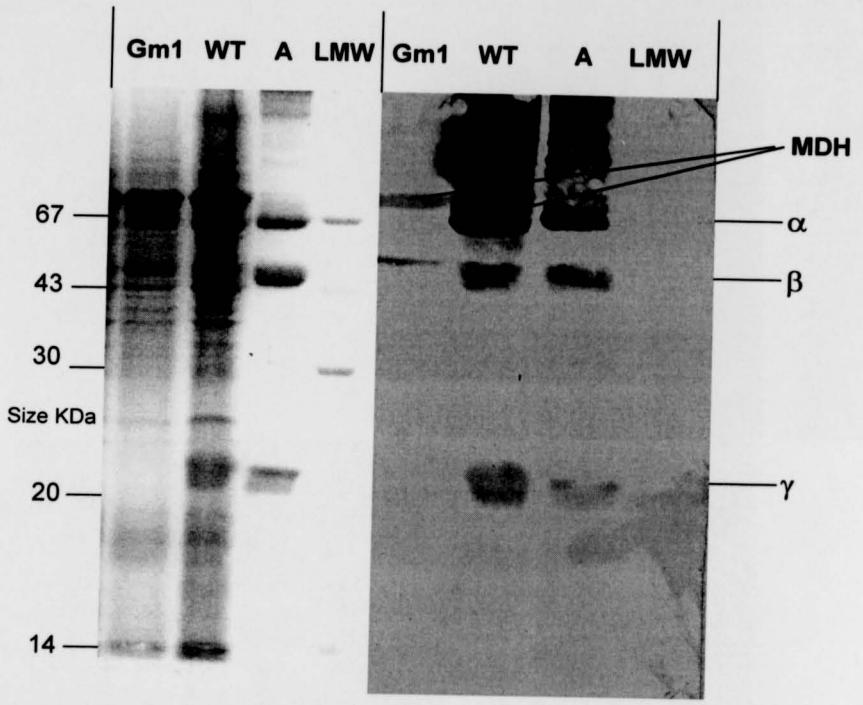

Fig 5.11 SDS PAGE and Western blot of *rpoN* mutant Gm1. Whole cell protein was extracted (see section 2.20.2) from *M. trichosporium* OB3b Gm1 and wild-type (WT) strains grown on low copper MS medium containing 0.05 % Glutamine (w/v). Protein concentrations were estimated using the BioRad assay. Approximately 90μg of the total cell protein was loaded per lane on a 12% SDS-PAGE gel. The left hand half was stained with Coomassie Brilliant Blue (panel A) and the right hand half left unstained. This was electroblotted onto nitrocellulose membrane and challenged with sMMO-hydroxylase specific rabbit IgG (panel B). sMMO hydroxylase (Protein A) was a gift from Sue Slade. Size standards were Low Molecular Weight Markers (LMW) from Amersham Pharmacia. The α, β, γ subunits of the sMMO hydroxylase and the large subunit of methanol dehydrogenase (MDH) are labelled.

The presence of sMMO transcripts was tested using RT-PCR. Primers specific for the *mmoX* gene (206F: ATCGCBAARGAATAYGCSCG; 886R: ACCCANGGCTCGACYTTGAA), encoding the α-subunit of the sMMO hydroxylase component were obtained from Marc Dumont (J.C. Murrell laboratory, University of Warwick). RNA was extracted from low copper grown batch cultures of wild-type and Gm1 mutants as described in section 2.17 and reverse transcribed using the primer 886R (see section 2.18). *mmoX*-specific PCR was then performed using primers 206F and 886R to give a product of approximately 750 bp (annealing temperature 60°C). The results clearly show that under the conditions tested, the

wild-type strain transcribes *mmoX*, whilst transcription is absent from the RNA extracted from three independent cultures of the Gm1 mutant (Figure 5.12).

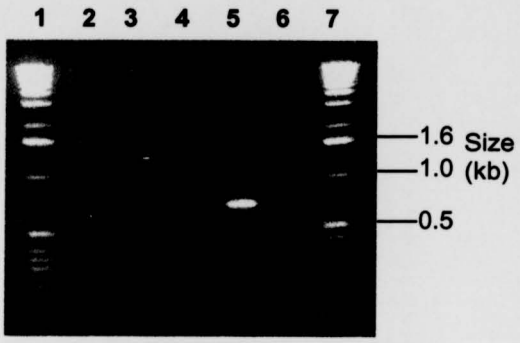

Fig 5.12 *mmoX*-specific RT-PCR of wild-type and Gm1 *M. trichosporium* OB3b strains.
RT-PCR was performed on total RNA extracted from the wild-type and Gm1 strains grown on MS medium containing 0.05 % (w/v) glutamine as described in section 2.?. Primers used were 206F:ATCGCBAARGAATAYGCSCG and 886R:ACCCANGGCTCGACYTTGAA; Products were analysed on a 2% TBE agarose gel. Lanes: 1, 1kb marker; 2, WT cDNA; 3, Gm1 cDNA; 4, Gm1 cDNA; 5, WT DNA positive; 6, Negative control; 7, 1kb marker. All RNA preparations were tested for contaminating DNA using *mmoX* PCR primers and were negative in all cases. The RNA used in lanes 3 and 4 originated from independent cultures and this result was confirmed using a further 2 independent cultures.

When the sMMO activity assays, SDS-PAGE, Western blots and RT-PCR data are considered as a whole, it is clear that the mutation in the *rpoN* gene abolishes *mmo* activity at the level of transcription. The product of the *rpoN* gene is the alternative sigma factor, σ^{54} (σ^{N}), and it is the loss of this protein, which directly affects the transcription of the *mmo* genes.

5.3.4 Growth of strain Gm1 on low copper medium

A mutation in the *rpoN* gene results in the loss of sMMO from the Gm1 strain. This enzyme is expressed at low copper to biomass ratios and thus it may expected that this mutant would be impaired in its ability to grow under copper limiting conditions. However, copper-limited cultures of Gm1 and wild-type OB3b using glutamine as sole source of nitrogen displayed almost identical doubling times (6 and 5.4 hours respectively), growth curves and final growth yields (figure 5.14D). These data indicated that the loss of sMMO does not impair growth in low copper batch cultures where presumably the cells are scavenging trace amounts of copper and using pMMO for growth.

5.3.5 Effects of *rpoN* knockout on nitrogen metabolism

It is known that *M. trichosporium* OB3b is capable of growth on several nitrogen sources including dinitrogen (N_2), nitrate and ammonium (Murrell and Dalton, 1983a,b). In many organisms, several facets of nitrogen metabolism are under the control of σ^N (σ^{54}) (For reviews see Merrick *et al.*, 1993; Merrick & Edwards, 1995). Therefore, in order to assess the role that σ^N plays in the regulation of these processes in *M. trichosporium* OB3b, an investigation of the ability of the *rpoN*⁻ strain Gm1 to grow on several nitrogen sources was tested.

In contrast to the wild-type strain, strain Gm1 was unable to grow under nitrogen fixing conditions (Figure 5.13) indicating that σ^N was required for the transcription of the nitrogen fixation genes in *M. trichosporium* OB3b. This enzyme converts nitrogen into ammonia, which becomes assimilated into cell biomass. It is therefore possible that the inability of the Gm1 strain to use N_2 as a nitrogen source is actually due to an inability to assimilate ammonia.

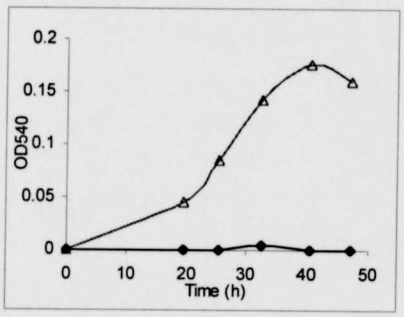

Figure 5.13 Growth of Wild-Type and Gm1 strains of *Methylosinus trichosporium* OB3b under nitrogen fixing conditions. Wild-type: grey triangles; Gm1: black diamonds. Both cultures were inoculated with 200 µl from a thick culture (OD540~0.6) grown on MS medium including glutamine (0.05% w/v). This experiment was repeated three times to test reproducibility and similar results were observed.

strain Gm1 was originally isolated on NMS agar, but grew very poorly on this medium. As NMS agar may contain trace amounts of alternative nitrogen sources, the ability of strain Gm1 to utilise nitrate (10 mM) as a sole nitrogen source in liquid NMS medium was tested. These growth curves conclusively show that the Gm1 strain cannot use nitrate as a sole nitrogen source (Figure 5.14A). However, the product of nitrate reduction by nitrate reductase and nitrite reductase is ammonia.

Therefore, a defect in the ammonia assimilation process may also be the cause of an inability to use nitrate as nitrogen source.

Previous work by Murrell and Dalton (1983b) had shown that *M. trichosporium* OB3b possessed both glutamine synthetase (GS) and glutamate synthase (GOGAT). These enzymes enable assimilation of ammonia by conversion to glutamine (GS) and then glutamate (GOGAT). Cell extracts from cells grown with nitrate (10 mM), ammonium (18 mM) and N_2 all possessed similar GS and GOGAT activities (Murrell and Dalton, 1983b). The absence of glutamate dehydrogenase was noted and indicated that *M. trichosporium* OB3b assimilated ammonia solely via the GS/GOGAT pathway. Thus, the observation in this study that strain Gm1 can utilise ammonia (18 mM) and glutamine (39 mM) as sole nitrogen sources at growth rates similar to the wild-type strain (Fig. 5.14B,C) indicates that its GS/GOGAT pathway is intact. Thus, the σ^N of *M. trichosporium* OB3b is probably required for the initiation of transcription of the enzyme systems responsible for nitrogen fixation and assimilation of nitrate but not the GS/GOGAT pathway.

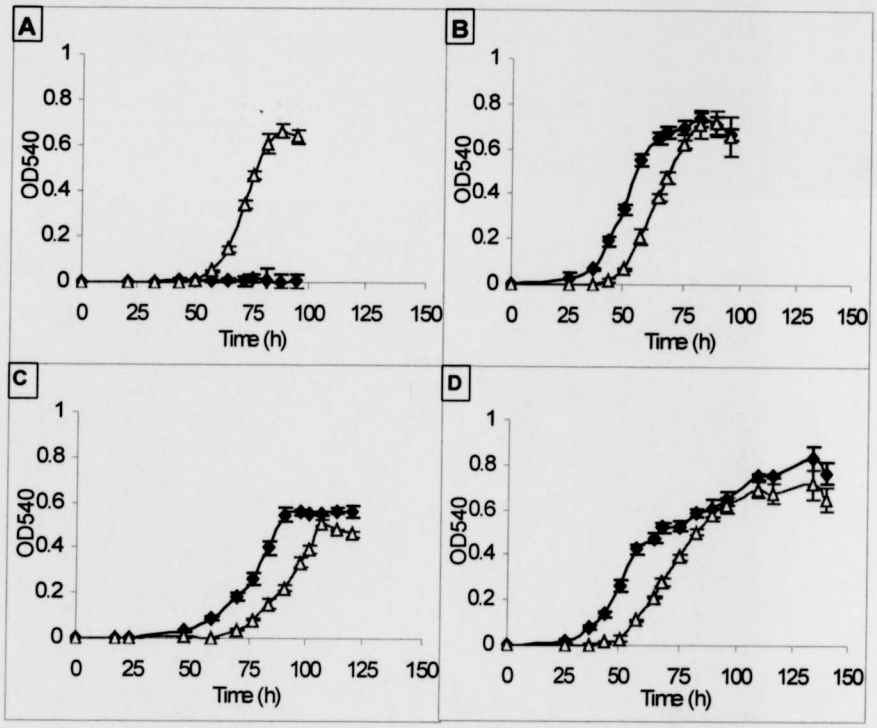

Figure 5.14 Growth curves of wild-type and Gm1 strains of *M. trichosporium* OB3b.
A: NMS; B: MS + Glutamine (0.05 %) (w/v); C: AMS; D: MS + Glutamine (0.05 %) (w/v) – low copper. Wild-type is represented by open grey triangles and the Gm1 strain by black diamonds. Growth curves were conducted as triplicate 50 ml batch cultures, standard deviation of the three values are shown (bars).

5.4 Investigation of SacB based vectors for the production of knockout mutants in *Methylosinus trichosporium* OB3b

To date, the narrow host range pBR329*mob* based suicide vectors have only twice been successfully used in *M. trichosporium* OB3b. Therefore, interruption of the desired gene is still a difficult process in this organism. In an attempt to increase the probability of obtaining the desired double crossover mutant, the possibility of using a SacB based system was investigated. This system relies on the positive selection of double recombinants using sucrose as the suicide substrate for the SacB gene. The gene encodes the enzyme levan sucrase which confers sucrose sensitivity on many strains of Gram negative and Gram positive bacteria (Schäfer *et al.*, 1994; Quandt and Hynes, 1993) and is a useful tool for detecting rare double crossover events.

5.4.1 Construction of pGPS104Gm

The vector used for this experiment was pK18*mob*SacB, it is a narrow-host range Kanamycin resistant plasmid containing an RP4*mob* determinant (Schäfer *et al.*, 1994). The Gm^R cassette from p34S-Gm was inserted into the *Bgl*II site in the centre of the *rpoN* gene of *Methylosinus trichosporium* OB3b to create pGPS519Gm. The whole *rpoN* cluster was excised on a *Hin*dIII fragment from pGPS519Gm and ligated into the *Hin*dIII site within the *lacZ* gene of pK18*mob*SacB to create pGPS104Gm (Figure 5.15).

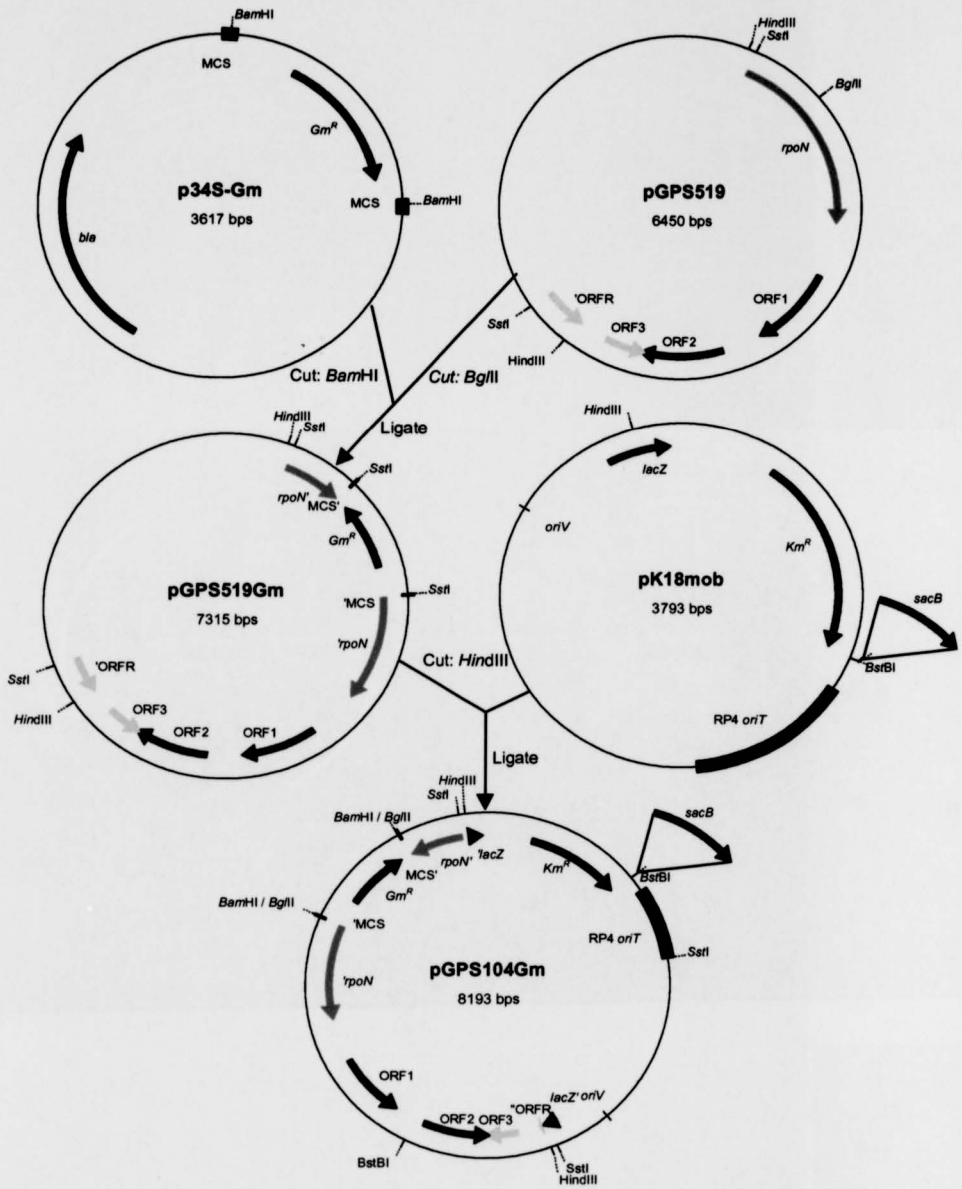

Figure 5.15 Construction of pGPS104Gm. Briefly, a Gentamycin cassette from p34S-Gm into a *Bgl*II site within pGPS519 to give pGPS519Gm. The *rpoN*-Gm fragment is then excised from pGS519Gm by digestion with *Hin*dIII before ligation into pK18*mob*SacB to create pGPS104Gm.

5.4.2 Conjugation of pGPS104Gm into *Methylosinus trichosporium* OB3b

After transfer of pGPS104Gm into *E.coli* S17-1, it was conjugated with *Methylosinus trichosporium* OB3b. Selection of exconjugants was on NMS agar plates containing gentamycin (5 μgml^{-1}). A total of 312 gentamycin-resistant exconjugants were obtained from this experiment (frequency 3.12×10^{-8}). When streaked onto plates containing both gentamycin (5 μgml^{-1}) and kanamycin (10 μgml^{-1}) all 312 grew, indicating the maintenance of the plasmid backbone in the chromosome of these strains i.e. a single crossover event had occured.

One of these strains, Gm2, was analysed by PCR (Figure 5.8) and Southern hybridisation (Figure 5.16A). This analysis showed that the Gm2 strain was a single recombinant inserted into the chromosome in orientation 2 (Figure 5.16B,C). Its ability to oxidise naphthalene was also tested and showed its possession of sMMO activity on low copper plates. It was also capable of growth on NMS, providing further evidence that the Gm1 strain was not a single recombinant strain.

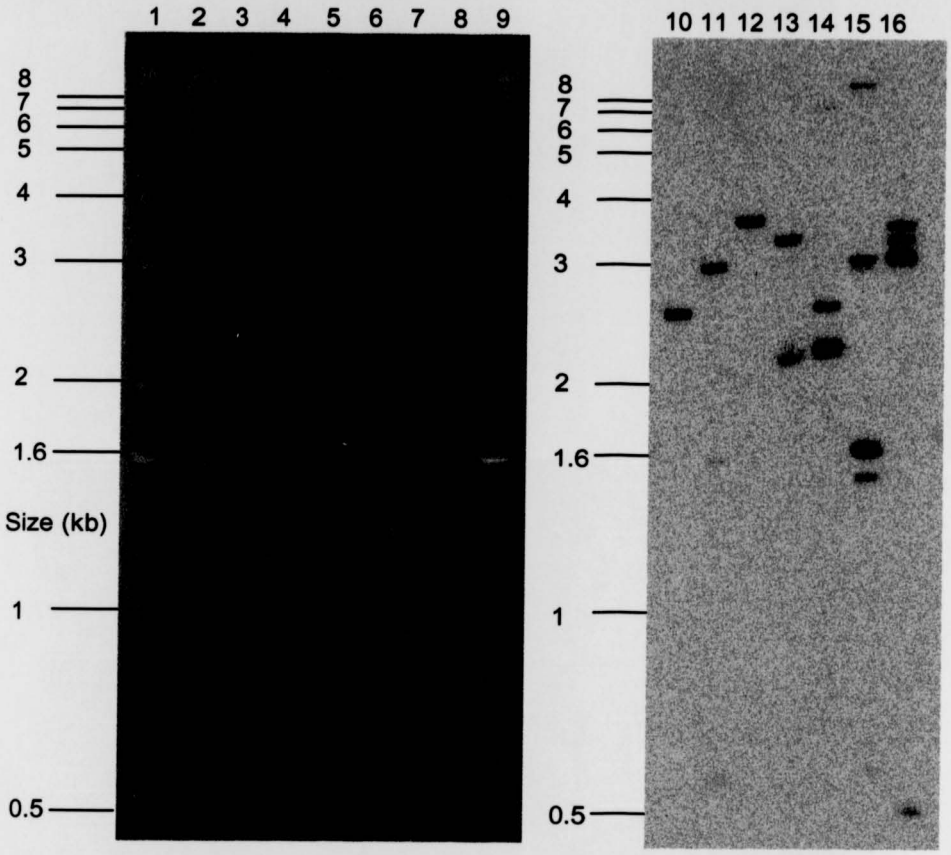

Figure 5.16 A: Restriction digest and Southern blot of Wild-type and Gm2 strain of *Methylosinus trichosporium* OB3b probed with *rpoN* gene from *Methylosinus trichosporium* OB3b. Lanes: 1, 1kb ladder; 2, WT *EcoR1*; 3, WT *SalI*; 4, WT *SstI;* 5, Gm1 *EcoR1* ; 6, Gm2 *EcoR1;* 7, Gm2 *SalI* ; 8, Gm2 *SstI* ; 9, 1kb; 10, WT *EcoR1*; 11, WT *SalI*; 12, WT *SstI;* 13, Gm1 *EcoR1* ; 14, Gm2 *EcoR1;* 15, Gm2 *SalI* ; 16, Gm2 *SstI*. **B: Table showing theoretical and actual hybridising fragments for the two possible orientations of single recombinants produced by pGPS104Gm.** When probed with rpoF6/OB3brpoR *rpoN* PCR product. Fragment sizes are shown in kb. Fragments assumed to be derived from incomplete digestion are illustrated in parentheses.

Theoretical: Orientation 1			Theoretical: Orientation 2			Observed: Strain Gm2		
EcoR1	SalI	SstI	EcoR1	SalI	SstI	EcoR1	SalI	SstI
3.6	>1.5	3.8	2.6	1.6	3.8	[7]	[>8]	3.8
1.3	1.3	3.5	2.2	1.4	3.1	2.6	[3]	[3.5]
	0.5	0.5		>0.5	0.2	2.2	1.6	3.1
							1.4	0.5
								0.6

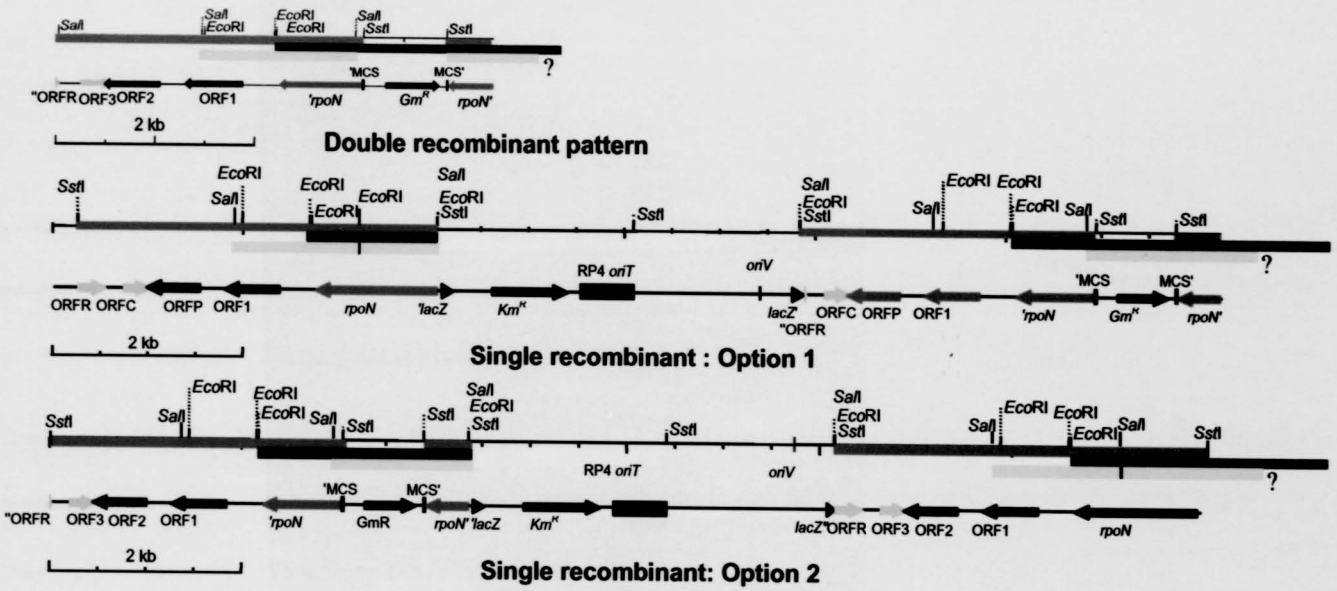

Figure 5.16 C Genetic organisation of double and single recombination events after homologous recombination between the *rpoN* gene from pGPS104Gm And the chromosome of *Methylosinus trchosporium* OB3b. Fragments expected to hybridise to OB3b *rpoN* probe are hghlighted in red (*Sst*I), blue (*Eco*RI) and Yellow (*Sal*I).

5.4.3 Selection of double recombinants

As mentioned above, the *sacB* gene product encodes the levan sucrase enzyme. Strains containing this gene become incapable of growth in medium containing 10 % (w/v) sucrose (Quandt and Hynes, 1994; Schäfer *et al.*, 1993). Thus, the Gm2 single recombinant strain of *M. trichosporium* OB3b which contains the plasmid backbone of pGPS104Gm inserted by a single recombinant event into its chromosome should be incapable of growth on medium containing 10 % sucrose (w/v). However, a double crossover event would result in the loss of the plasmid backbone, including the *sacB* gene from the chromosome and thus confer sucrose resistance on the Gm2 strain. In order to select for such an event, strain Gm2 was inoculated onto MS agar containing 0.05 % glutamine (w/v), 10 % sucrose (w/v) and gentamycin ($5\mu gml^{-1}$). Approximately 20 colonies were present on the sucrose containing agar after 10 days. The colonies capable of growth on this medium should have lost the plasmid backbone from their chromosome and have a $Sucrose^{R}Gm^{R}Km^{S}$ phenotype. However, all 20 of the sucrose resistant strains still possessed a Km^{R} phenotype, indicating the presence of the plasmid backbone in the chromosome of these strains. The sucrose-resistant strains which retained the Km^{R} marker presumably contained a mutation in the *sacB* gene rendering it inactive whilst retaining the Km^{R} marker. A similar phenomenon was observed by Quandt and Hynes, (1993), where 50 % of sucrose-resistant clones retained the plasmid derived antibiotic marker.

During the writing of this thesis Csaki *et al.*, (2001) reported the use of a *sacB* derived plasmid for the inactivation of the membrane-bound hydrogenase gene from *Methylococcus capsulatus* Bath. These workers used a selection method where late-exponential phase liquid cultures of the single recombinant strain were incubated in the presence of sucrose (10 % w/v) for 48 hours to enrich for double recombinants. Double recombinants were then screened for sensitivity to plasmid-derived (gentamycin) antibiotic resistance. Replication of this method may have allowed the inactivation of the *rpoN* gene of *Methylosinus trichosporium* OB3b, but further attempts were not possible due to the time-frame of this project. However, future gene knockout experiments in this organism may be aided by the method reported by Csaki *et al.*, (2001).

5.5 An examination of the role of σ^N from *Methylococcus capsulatus* Bath

To examine the role of σ^N in *Methylococcus capsulatus* Bath and compare the phenotypes with those of an *rpoN* $^-$ mutant of *Methylosinus trichosporium* OB3b, attempts were made to mutate the *rpoN* gene of *Methylococcus capsulatus* Bath. The sequence of the *rpoN* gene of *M. capsulatus* Bath was a kind gift from Oivind Larsen, University of Bergen, Norway.

5.5.1 Construction and conjugation of knockout vectors – pBR329*mob* backbone

The entire *rpoN* gene was amplified, by PCR, using primers rpoMCF: AAA ACT GCA GAT GAA ACA ATC ACT GCA AC and rpoMcR: AAA ACT GCA GTT AGA ACA ACT GCT TTC TTT CG with annealing temperature of 65 °C (from the chromosome of *Methylococcus capsulatus* Bath), and cloned into the pCRTOPO-2.1 vector to create pGPS110. A 429 bp *Sal*I deletion was created in *rpoN* and replaced by either a Gm^R or Sm^R/Sp^R cassette from p34S-Gm and p34S-Sm/Sp, respectively. These fragments were then excised using *Eco*R1 and placed into pBR329*mob* to create pGPS111 (Sm/Sp) and pGPS113 (Gm) (Figure 5.17).

Repeated attempts to conjugate these constructs into *Methylococcus capsulatus* Bath with selection on NMS Gm (5 $\mu g ml^{-1}$) or Sm (20 $\mu g ml^{-1}$) failed to produce any exconjugants. One reason for the lack of exconjugants could have been that selection on NMS was impossible due to the inability of and *rpoN* $^-$ mutant to utilise nitrate as a sole nitrogen source. However, σ^N does control the nitrate/ nitrite reductase system in *Methylosinus trichosporium* OB3b, and it was possible to gain exconjugants from that organism.

It should be noted that these marker-exchange experiments were performed before the *rpoN* $^-$ strain of *Methylosinus trichosporium* OB3b had been constructed. Unfortunately, within the time scale of this project it was not possible to repeat these experiments with selection on AMS or MS + glutamine (0.05 % w/v). Utilisation

of these substrates should be unaffected by the knockout of *rpoN*, and represents the best avenue for future experiments.

5.5.2 Construction and conjugation of knockout vectors – pK18*mob*SacB backbone

A personal communication by Robert Csaki (University of Szeged, Hungary) indicated that it was possible to use SacB based vectors to produce knockout mutants in *Methylococcus capsulatus* Bath. Indeed, during the writing of this thesis Csaki *et al.*, (2001) reported the successful use of this technique to produce marker exchange mutants of the membrane-bound hydrogenase genes in *M. capsulatus* Bath. This method was thus investigated in an attempt to knockout the *rpoN* gene of *Methylococcus capsulatus* Bath.

To create a pK18*mob*SacB based knockout vector, an *Eco*R1 fragment was excised from pGPS113 and placed into the *Eco*R1 site within the *lacZ* gene of pK18*mob*SacB to create pGPS114 (Figure 5.18). Conjugation of this vector into *Methylococcus capsulatus* Bath failed to produce any exconjugants with selection on NMS containing Km (10 μgml^{-1}). Selection on Kanamycin containing plates would preferentially select for single crossover mutants thereby avoiding the possible problem caused by selection on NMS agar.

These results indicated that the failure of both pBR329*mob* and pK18*mob* based vectors to produce exconjugants is unlikely to be solely the result of selection on NMS. Another explanation for the failure of this experiment could be the relatively short lengths of the flanking regions around the deletion (270 and 700 bp). A study by Quandt and Hynes (1993) on the production of single and double recombinant mutants indicated that increasing the length of flanking DNA (>1kb) increased the amount of double recombinant mutants over single recombinants in *Rhizobium leguminosarum*. However, single recombinants were still obtained using flanking regions of 700 bp. It is therefore possible that the 270 bp flanking region used in this experiment was too short to allow adequate binding for recombination to occur. Once again, the time scale of this project did not allow the construction of vectors with larger stretches of DNA flanking the *rpoN* gene. Such an approach may allow inactivation of this gene and thus provide an interesting comparison between the role of σ^N in these two methanotrophs.

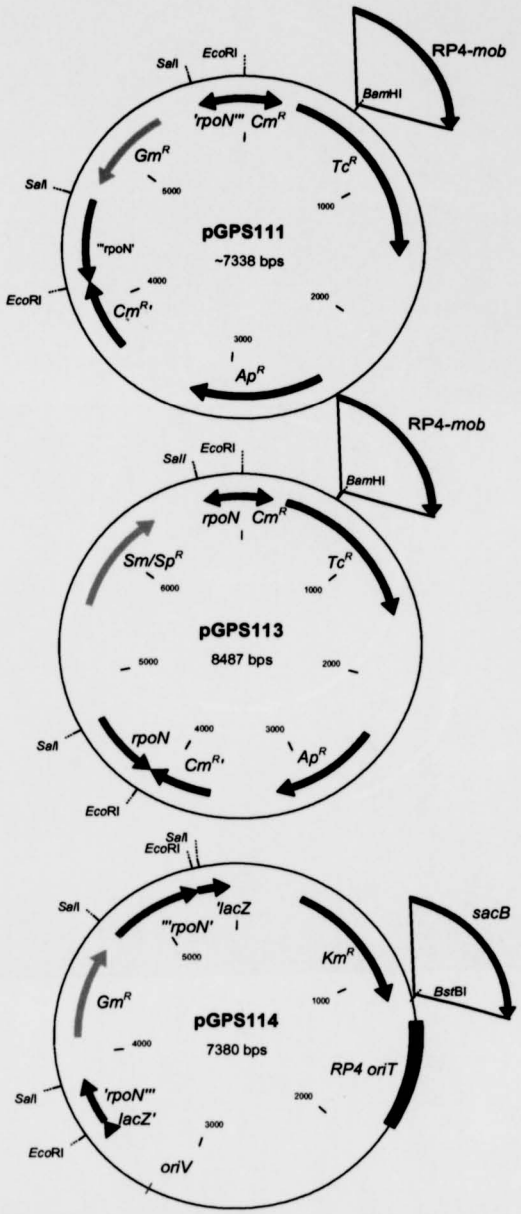

Figure 5.17 Vectors constructed for the knockout of the *rpoN* gene from *Methylococcus capsulatus* Bath.

5.6 Discussion

A previous study by Allan Nielsen (1997) revealed the presence of a σ^N-type promoter corresponding to the transcriptional start site of the *mmo* operon from *Methylosinus trichosporium* OB3b. In this chapter, data have been presented which shows the presence of an *rpoN* gene and the requirement of its gene product for at least three processes: 1. expression of the soluble methane monooxygenase enzyme; 2. growth on nitrate as a sole source of nitrogen; 3. nitrogen fixation.

5.6.1 The *rpoN* gene from *Methylosinus trichosporium* OB3b

Using the *Sinorhizobium meliloti* 1021 *rpoN* gene as a probe, the *rpoN* gene from *Methylosinus trichosporium* OB3b was isolated. Phylogenetic analysis clearly identified the *rpoN* of *M. trichosporium* OB3b as most closely related to the *rpoN* of other members of the α-subclass of the *Proteobacteria,* especially the family *Rhizobiaceae.*

In most organisms analysed to date, the organisation of ORFs linked to the *rpoN* gene is well conserved (Merrick, 1993). Downstream (3') of the *rpoN* gene from *Methylosinus trichosporium* OB3b lie two complete open reading frames: ORF1 and ORF2; which are often found in association with *rpoN* genes in many other bacteria (Merrick, 1993; Powell *et al.,* 1995). The genetic linkage of these genes in many organisms, and now in *M. trichosporium* OB3b, seems to support the proposal that these genes are involved in co-regulation of σ^N-dependent operons (Merrick 1993; Michiels *et al.,*1998a; Powell *et al.,* 1995). However, their role seems to differ between organisms and a clear definition of their function has yet to be achieved.

Analysis of the DNA sequence between *rpoN* and ORF1 revealed a possible Shine-Dalgarno and stem-loop terminator sequence in the region between the stop-codon of *rpoN* and the start codon of ORF1. However, no putative terminators were found between ORFs 1 and 2 indicating that ORF1 and ORF2 may be transcribed independently from *rpoN*. Therefore, an insertional mutation in *rpoN* probably would not have a polar effect on ORFs 1 and 2.

5.6.2 Mutation of *rpoN*: effects on nitrogen metabolism

Mutation of the *rpoN* (formerly *glnF* and *ntrA*) gene of *M. trichosporium* OB3b has two major effects on nitrogen metabolism. The Gm1 strain is not able to grow under nitrogen fixing conditions or to utilise nitrate as its sole nitrogen source. The involvement of σ^N with nitrogen metabolism has been known for many years. It acts in the regulation of nitrogen fixation genes in *Klebsiella pneumoniae* (Merrick *et al.*, 1985), *Sinorhizobium meliloti* (Ronson *et al.*, 1987), *Rhizobium leguminosarum* and *Azotobacter vinelandii,* among others (Merrick *et al.*, 1993). The data presented here allow *Methylosinus trichosporium* OB3b to be added to this list.

In addition to its role in regulation of nitrogen fixation, σ^N, can also regulate the use of several other inorganic nitrogen sources including nitrate and urea (Merrick, 1993). A mutation in the *rpoN* gene of *Sinorhizobium meliloti* and *Ralstonia eutrophus* (formerly *Alcaligenes eutrophus*) results in an inability to use potassium nitrate as a sole nitrogen source (Ronson *et al.*, 1987; Römmermann *et al.*, 1989). However, nitrate assimilation has been studied at the genetic level in only a few cases. In *Klebsiella pneumoniae* and *Azotobacter vinelandii,* the nitrate assimilation pathway is regulated in both a σ^N-dependent manner in response to ammonium levels and is also induced by nitrate or nitrite in a pathway specific manner. This second mechanism involves the anti-terminator NasR in *K. pneumoniae* (Wu *et al.*, 1999; Goldman *et al.*, 1994) and the unusual two-component NasS/NasT system in *A. vinelandii* (Moreno-Vivian, 1999; Lin and Stewart, 1998). The data presented here indicate that nitrate assimilation in *Methylosinus trichosporium* OB3b is dependent on σ^N, adding nitrate to N_2 in the list of σ^N-regulated systems in this organism.

The fixation of N_2 and the reduction of nitrate both result in the production of ammonia, which is assimilated solely by the glutamine synthetase (GS)/ glutamate synthase (GOGAT) pathway in *Methylosinus trichosporium* OB3b (Murrell and Dalton, 1983). Although in some organisms this is the only pathway for the assimilation of ammonia, an alternative route exists for the assimilation of ammonia. In many organisms, glutamate dehydrogenase (GDH), which possesses a high K_m for ammonia (1mM), is responsible for assimilation of ammonia at high concentrations and the GS/GOGAT pathway is typically responsible for assimilation of ammonia levels lower than 0.1mM (Merrick and Edwards, 1995). Thus, one could propose that

the ability of Gm1 to grow on 18 mM ammonia is due to an intact GDH (or alanine dehydrogenase, ADH) pathway whose regulation is σ^N-independent. However, previous work by Murrell and Dalton (1983) failed to detect GDH or ADH activity under such conditions. Thus, it appears that *M. trichosporium* OB3b possesses a constitutive, σ^N-independent, GS activity.

Four types of glutamine synthetase enzyme have been described: GSI encoded by *glnA*, GSII encoded by *glnII*, GSIII encoded by *glnN* and a different GSIII encoded by *glnT* (Merrick and Edwards, 1995)(See section 1.8.2). The classic picture of nitrogen control was established chiefly by pioneering studies conducted in the enteric bacteria, where GS expression is repressed by high intracellular levels of ammonia (Reviewed in Reitzer and Schneider, 2001; Merrick and Edwards, 1995). In these organisms, *glnA* transcription occurs from two promoters, *glnAp1* and *glnAp2*, and is activated during of nitrogen limitation by NtrC in a σ^N-dependent manner from *glnAp2*. In times of nitrogen sufficency, transcription occurs at a basal level from *glnAp1* by $E\sigma^{70}$. However, regulation of *glnA* is very different in many other bacteria. Indeed in *Rhizobium leguminosarum, Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*), *glnA* lies in a *glnBA* operon and appears to be expressed constitutively (De Bruijn *et al.,* 1989; Chiurazzi and Iaccarino, 1990). This genetic organisation is also present in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* where *glnB* and *glnA* are expressed under conditions of ammonia limitation and repressed in the presence of excess ammonia in an NtrC-dependent, but σ^N-independent manner (Zinchenko, *et al.,* 1994; Foster-Harnett and Kranz, 1994). Several species of the family *Rhizobiaceae* contain at least two distinct forms of GS. The best studied of these is *Sinorhizobium meliloti,* which contains three forms of the enzyme; GSI (*glnA*), GSII (*glnII*) and GSIII (*glnT*). *glnA* is constitutively transcribed whilst *glnII* and *glnT* are transcribed under nitrogen limitation by σ^N and NtrC (Shatters *et al.,* 1993; De Bruijn *et al.*, 1989). Thus it appears possible that the ability of strain Gm1 to utilise high ammonia levels could result from one or more GS enzymes.

The reaction catalysed by GS results in the production of glutamine from ammonia and glutamate. In order for this glutamine to be assimilated into cell biomass, it must be converted into glutamate by the GOGAT enzyme. A GOGAT⁻ strain would be unable to utilise ammonia or nitrate as a sole nitrogen source, or

assimilate fixed nitrogen, as exemplified in *Azospirillum brasilense* and *Sinorhizobium meliloti* (Mandal and Ghosh, 1993; Lewis *et al.*, 1990). Therefore, the ability of both the wild-type and Gm1 strains of *M. trichosporium* OB3b to utilise glutamine as a sole nitrogen source indicates the presence of a functional GOGAT enzyme in strain Gm1.

The creation of an *rpoN* mutant of *Methylosinus trichosporium* has revealed the presence of a nitrogen control circuit in which the assimilation of nitrate and fixation of nitrogen are reliant on σ^N. It has also been shown that the constitutive GS activity observed by Murrell and Dalton (1983b) may be σ^N-independent. These data have provided an invaluable insight into the regulation of nitrogen metabolism in this widely distributed free-living nitrogen fixing organism. However, σ^N is not exclusively involved in the regulation of nitrogen metabolism genes, and its role in methane oxidation is discussed in section 5.6.3.

5.6.3 Mutation of *rpoN*: effects on *mmo* expression

In this chapter the phenotypes of an *rpoN* mutant were examined. As discussed in section 5.6.2 this mutation impaired several aspects of nitrogen metabolism. The σ^N protein is also known to be involved in the regulation of a wide variety of genes involved in a diverse range of functions including hydrogen oxidation (Römmermann *et al.*, 1989), C4-dicarboxylic acid transport (Ronson *et al.*, 1987), acetoin catabolism (Krüger *et al.*, 1992, 1994) and toluene/*o*-xylene metabolism (Arenghi *et al.*, 1999). The oxidation of methane via the soluble methane monooxygenase of *Methylosinus trichosporium* OB3b can now be added to this list.

Disruption of *rpoN* results in a strain of *M. trichosporium* OB3b that is unable to produce the soluble methane monooxygenase enzyme. RT-PCR experiments revealed that this strain is no longer capable of transcriptional initiation from the σ^N dependent promoter 5' of *mmoX*. The confirmation of a role for σ^N in the initiation of transcription of the *mmo* operon from *Methylosinus trichosporium* OB3b, coupled with the identification of σ^N promoters upstream of the *mmo* operons of *Methylocystis* sp. strain M (McDonald *et al.*, 1997), *Methylomonas* sp. strains KSPII and KSWII (Shigematsu *et al.*, 1999) and a poor consensus promoter in *Methylococcus capsulatus* Bath implies that this mechanism of regulation may not be confined to *Methylosinus trichosporium* OB3b (Nielsen *et al.*, 1996).

In addition to the σ^N type promoter located 5' of *mmoX*, a σ^{70}-type promoter sequence lies between *mmoX* and *mmoY* in *Methylosinus trichosporium* OB3b (Nielsen *et al.*, 1997). Primer extension and northern analysis of sMMO transcription suggest that this promoter may be functional *in vivo* (Nielsen *et al.*, 1997). Western blot analysis of the Gm1 strain may have allowed the functionality of this promoter to be established. If the Gm1 mutant possessed both β and γ-subunits of the hydroxylase component whilst lacking the α-subunit, it would have been possible to imply that this indicated transcription from this second promoter. Western blots of cell extracts from the Gm1 mutant failed to detect the γ or α-subunits but contained the β-subunit. It is possible that transcription from the σ^{70} promoter could result in expression of both β and γ-subunits, but that the γ–subunit is unstable in the absence of the α-subunit and so is absent from these blots. However, the latter explanation is unlikely since Lloyd (1997) observed that the β and γ–subunits of sMMO in *M. trichosporium* OB3b were present in cell extracts from the *mmoX*$^-$ mutant (F) of *Methylosinus trichosporium* OB3b, grown on low copper medium. It seems more likely that the failure to detect the γ-subunit is due to the levels of this protein falling below the detection threshold in this experiment, suggesting that the σ^{70} promoter may be active under these conditions. The presence of lower levels of the β-subunit in protein extracts from the Gm1 strain than the wild-type suggests that transcription from this promoter is at a much lower level than from the *mmoX* promoter. However, in order to confirm these possibilities, Western blots using antibody against each individual subunit must be performed to determine the levels of the sMMO subunits. RT-PCR and Northern analysis could also be used to assess the levels of *mmoY* transcripts in the Gm1 mutant strain in response to the concentrations of copper ions.

The *mmo* genes are expressed at low copper concentrations, typically below 0.25μM (Stanley *et al.*, 1983, Nielsen *et al.*, 1997,1996), whereas the *pmo* operon is expressed chiefly at high copper concentrations, co-purifies with 16 copper ions per enzyme complex and is believed to contain copper in its active site (Nguyen *et al.*, 1998, 1994; Dispirito *et al.*, 1998). Thus, it may be expected that an organism containing a mutation affecting the *mmo* system would be impaired in its growth in low copper media. A strain of *Methylosinus trichosporium* OB3b, mutated in the *mmoX* gene (mutant F), was reported by Martin & Murrell (1997) to be incapable of growth in a copper depleted batch culture medium. However, Lloyd (1997) observed

growth of mutant F in the same medium. Batch cultures of *M. trichosporium* OB3b mutant Gm1 were also capable of growth in low copper medium, with a growth doubling time almost identical to the wild-type. In the case of both mutant Gm1 and Mutant F these cultures did not express sMMO activity under low-copper conditions. Growth of these mutant cultures probably occurred due to their ability to scavenge trace amounts of copper from the medium, allowing pMMO expression.

The pMMO enzyme system allows faster growth rates than the sMMO system (Stanley *et al.,* 1983; Leak and Dalton, 1986). This is illustrated in this study where wild-type *M. trichosporium* OB3b had a doubling time of 4.6 h on 'normal' copper levels and 5.4 h under copper limitation. However, growth of the pMMO-only strain, Gm1, was only marginally slower under copper limitation than the wild-type strain (expressing sMMO). This observation probably reflects both the ability of *M. trichosporium* OB3b to scavenge copper from its environment and the slower growth rates enabled by sMMO in the wild-type strain and pMMO in strain Gm1 under copper stress. Indeed, the removal of copper from the growth medium of methanotrophs containing only the pMMO enzyme system results in poor growth of, for example, *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP (Hanson and Hanson, 1996).

The data presented in this chapter establish a link between the σ^N protein and transcriptional regulation of the soluble methane monooxygenase genes (*mmo*), nitrate/nitrite assimilation and nitrogen fixation in *Methylosinus trichosporium* OB3b. It is the first time a sigma factor has been identified in this organism and is a major breakthrough in the study of the "copper-switch" and nitrogen metabolism. The mechanism of transcription from σ^N-dependent promoters has been studied in great detail (Reviewed by Buck *et al.,* 2000) and has shown that initiation of transcription from σ^N-type promoters requires an activator protein in all known cases. It is the search for such an activator of transcription for the *mmo* operon of *Methylosinus trichosporium* OB3b which forms the basis for the next chapter.

Chapter 6

Sequence analysis and partial characterisation of *mmoR* and *groEL* genes from *Methylosinus trichosporium* OB3b

6.1 Introduction

The mechanisms controlling expression of the methane monooxygenase genes in response to copper, the copper switch, has long been a topic of speculation since its discovery by Stanley *et al.*, (1983). In recent years, the picture has become clearer. Nielsen *et al.*, (1997) showed that transcription of the soluble (sMMO) and particulate methane monooxygenase (pMMO) operons was reciprocal, with expression at low and high copper levels respectively. He also identified a probable promoter element upstream of the *mmo* operon, encoding sMMO, which belonged to the σ^N family of promoters in *Methylosinus trichosporium* OB3b (See section 1.5.1). More recently, putative σ^{70}-type promoters have been identified 5' of the *pmo*, encoding pMMO, of *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (Gilbert *et al.*, 2000; Chapter 3).

Further insights were gained from the results presented in chapter 5 of this thesis. An insertion mutant (Gm1) in the *rpoN* gene, encoding the σ^N-sigma factor, resulted in a strain that was unable to express the sMMO enzyme. Thus, it appears that the *mmo* operon is under the control of σ^N. This mode of transcriptional activation requires an additional factor to σ^N. These proteins are called enhancer-binding-proteins (EBPs) due to the fact that they facilitate initiation of transcription from remote enhancer sequences, named upstream activator sequences (UAS), which lie upstream from the promoter to which σ^N binds (Reviewed in Morrett and Segovia, 1993; Section 1.7.2).

This chapter focuses on sequence analysis of two open-reading frames, one of which is a putative EBP for the *mmo* operon and describes the construcion of an insertional mutant in the EBP-like open reading frame and the subsequent phenotypic characterisation of this mutant.

6.2 Sequence analysis of an *acoR*-like putative transcriptional regulator and a *groEL* homologue

The genes encoding the soluble methane monooxygenase lie in a six gene operon in the chromosome of *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991). Recent sequencing by Ian McDonald, of the region upstream (5') from the first gene of this operon, *mmoX*, has revealed the presence of a further two open reading frames (figure 6.1).

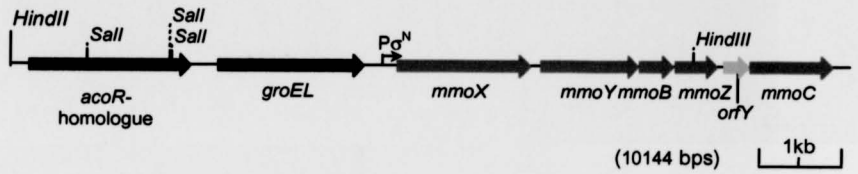

Figure 6.1 Genetic organisation of the *mmo* operon and upstream sequences.
$P\sigma^N$ refers to the sigma N promoter sequence identified by Nielsen *et al.*, (1997).

The first of the open reading frames is a member of the *nifA*-family of σ^N-dependent enhancer binding proteins (EBPs). Its derived amino acid sequence possesses 27 % identity to that of the *acoR* gene from *Ralstonia eutrophus* (formerly *Alcaligenes eutrophus*)(Krüger & Steinbüchel, 1992). The *acoR* gene product is a positive regulator of transcription of the acetoin catabolism operon (*acoXABC*). The genes of this operon encode the α and β subunits of the acetoin:2,6-dichlorophenolindophenol oxidoreductase (*acoAB*), and two other proteins of unknown function (*acoX, acoC*) and are transcribed by an *acoR*-dependent manner in response to acetoin levels (Krüger and Steinbüchel, 1992). Members of this family of transcriptional activator proteins are characterised by their conserved modular structure, comprising of an N-terminal variable effector region, a highly conserved central domain and DNA-binding C-terminal domain (Morrett and Segovia, 1993). An alignment of the central and C-terminal domains of the *M. trichosporium* OB3b *acoR*-like gene reveals striking homology with the central and C-terminal regions of 10 other members of this family of regulators (Figure 6.2, Table 6.1). The central domain contains the characteristic Walker A and B ATPase domains, a nucleotide-binding sequence and a conformational change switch motif (Morrett and Segovia, 1993). The C-terminal domain contains a putative DNA-binding helix-turn-helix motif.

Table 6.1 Amino acid identities between *mmoR* and 10 other EBPs exerting control over operons of diverse function. Sequences were aligned in a pairwise manner using the Blast2 Tool from NCBI with the BlastP database and Blosum62 matrix. Identities are expressed as percentage of identity with the entire 649 amino acids from MmoR. With 100% representing 649 identities from out of a possible 649.

Regulator	Main role	Organism	Identity to MmoR (%)	Reference (Accession Number)
AcoR	Acetoin catabolism	*Ralstonia eutrophus*	27	Krüger & Steinbüchel, (1992) (AAA21944.1)
DmpR	Phenol catabolism	*Pseudomonas putida*	25	Shingler *et al.*,(1993) (A47078)
TbuT	Toluene-3-monooxygenase	*Ralstonia pickettii*	24	Byrne & Olsen (1996) (AAC44567.1)
HbpR	2-hydroxybiphenyl metabolism	*Pseudomonas azelaica*	24	Jaspers *et al.*, (2000) (AAB57638.1)
DctD	C4-Dicarboxylate transport	*Rhizobium leguminosarum*	26	Ronson *et al.*, (1987) (P10046)
NifA	Nitrogen fixation	*Sinorhizobium meliloti*	16	Buikema *et al.*, (1985) (P03028)
PilR	Pilin assembly	*Pseudomonas aeruginosa*	16	Ishimoto *et al.*, (1992) (L22436)
NtrC	Nitrogen fixation	*Salmonella typhimurium*	18	Kustu, direct submission (S53024)
FlbD	Flagellar genes	*Caulobacter crescentus*	17	Ramakrishnan and Newton (1990) (P17899)
XylR	Xylene degradation	*Pseudomonas putida*	17	Inouye *et al.*, (1988) (NIPSRP)

A

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                                                                                Walker A
AcoR  Msitri  AGCGAELAELRACDAEWRDGVLETALRKASGLQERNIPILITGESGVGKD
AcoR  Raleut  PAETQVAMPDALAALTGGDAALQLQLQRAARLVDSPINLLIHGETGSGKE
DmpR  Pseput  LRTNLDKQEGQYYG-IGQTPAYQTVRNMMDKAAQGKVSVLLLGETGVGKE
TbuT  Ralpic  IAARLSNAPENSFGVVGISAGFNTVCHMVNKVAPTEATVLFLGESGVGKE
HbpR  Pseaze  ----------S---AVFKVLRHKTACVAETDATVLLLGESGSGKS
DctD  Rhileg  ----------LIGQTPVMENLRNILRHIADTDVDVLVAGETGSGKE
NifA  Sinmel  ----------GIIGESTALMTAVDTAKVMAETNSIVLLRGETGTGKE
PilR  Pseaer  ----------RLLGESPPMRALRNQIGKLARSQAPVYISGESGSGKE
NtrC  Saltyp  ----------DMIGEAPAMQDLFRIIGRLSRSSISVLINGESGTGKE
FlbD  Caucre  ----------MVVRDPAMEQVIKLADQVAPSEASILITGESGSGKE
XylR  Pseput  LRNRLKQYDGQYYG-IGHSPAYKRICETIDKAARGRVSVLLLGETGVGKE
                                                          ** * **

                                                    Switch motif
AcoR  Msitri  HLVRRLHAIGPRKDRPLVAINCAAIPRELIESELFGYEGGSFTGARAKGK
AcoR  Raleut  FLAKALHLASARRGGPFVAVNCAAIPETLIESELFGHLPNSFSGAGPRGK
DmpR  Pseput  VIARSVHLRSKRAAEPFVAVNCAAIPPDLIESELFGVEKGAFTGASQS-R
TbuT  Ralpic  VFANNLHRLSKRADGPFVAVNCASIPEHLMESELFGVERGGFTGATTS-R
HbpR  Pseaze  LIAREIHRLSNRADQAFVEVNCAAIPDQLIESELFGVERGAFTGATAT-R
DctD  Rhileg  VVAQILHQWSHRRKGNFVALNCGALPETVIESELFGHERGAFTGAQKR-R
NifA  Sinmel  CFAKLIHQHSTRQKKPFIKFNCPALSESLLESELFGHEKGAFTGAIAQ-R
PilR  Pseaer  LVARLIHEQGPRIERPFVPVNCGAIPSELMESEFFGHKKGSFTGAIED-K
NtrC  Saltyp  LVAHALHRHSPRAKAPFIALNMAAIPKDLIESELFGHEKGAFTGANTI-R
FlbD  Caucre  VMARYVHGKSRRAKAPFISVNCAAIPENLLESELFGHEKGAFTGAMAR-R
XylR  Pseput  VIARSVHLRSERAEQPFVAVNCAAIPPDLIESELFGVDKGAYTGAVNA-R
                    *    *       *          *** **       **

                          Walker B
AcoR  Msitri  PGKFVEADKGILFLDEIGDMAADLQATLLRVLDSSEVVPIGSSKPIRVDV
AcoR  Raleut  RGLIQEADGGTLFLDEIGDMPRELQSRLLRVLAEGEVLPVGAARPVPVRL
DmpR  Pseput  MGRFERADKGTIFLDEVIELSPRAQASLLRVLQEGELERVGDNRTRKIDV
TbuT  Ralpic  PGRFERADGGTLFLDEIGTLSLTAQGKLLRALQQGEIERVGDTRTRKVNV
HbpR  Pseaze  EGRFEAAHQGTLFLDEIATLSMTAQSKLLRVLQNGELERLGSNRTIHTSV
DctD  Rhileg  TGRIEHASGGTLFLDEIESMPAATQVKMLRVLEMREITPLGTNEVRPVNL
NifA  Sinmel  VGRFESANGGTLLLDEIGEIPPAFQAKLLRVIQEGEFERVGGTKTLKVDV
PilR  Pseaer  QGLFQAASGGTLFLDEVADLPMAMQVKLLRAIQEKAVRAVGGQQEVAVDV
NtrC  Saltyp  QGRFEQADGGTLFLDEIGDMPLDVQTRLLRVLADGQFYRVGGYAPVKVDV
FlbD  Caucre  IGKFEEADGGTLLLDEISEMDVRLQAKLLRAIQEREIDRVGGSKPVKVNI
XylR  Pseput  AGRFERANGGTIFLDEVIELTPRAQATLLRVLQEGELERVGGDRTRKVDV
                *    *  *   ***         *   **         *

AcoR  Msitri  RVVAATNRSLPEMVQKGTFRRDLYYRLNGVQLWLPPLRERPDRLRLLAHL
AcoR  Raleut  RVISATHHSLEQLVADGRFREDLYYRLNGARFTLPPLRAR-TDLDWLVRK
DmpR  Pseput  RVIAATHEDLAEAVKAGRFRADLYYRLNVFPVAIPALRERREDIPLLVEH
TbuT  Ralpic  RVIAATNVNLREAAKAGQFREDLFFRLNVFPIQVPPLRERRDDIPLMMNW
HbpR  Pseaze  RLIAATNADLKKAVQDGHFREDLYYRLNVFPIQIPPLRERRDDISLITSV
DctD  Rhileg  RVVAAAKIDLGDPAVRGDFREDLYYRLNVVTISIPPLRERRDDIPLLFSH
NifA  Sinmel  RLIFATNKDLEMAVQNGEFREDLYYRISGVPLILPPLRHRDGDIPLLARA
PilR  Pseaer  RILCATHKDLAAEVGAGRFRQDLYYRLNVIELRVPPLRERREDIPLLAER
NtrC  Saltyp  RIIAATHQNLERRVQEGKFREDLFHRLNVIRIHLPPLRERREDIPRLARH
FlbD  Caucre  RILATSNRDLAQAVKDGTFREDLLYRLNVVNLRLPPLRERPADVISLCEF
XylR  Pseput  RLITATNENLEEAVKMGRFRADLFFRLNVFPVHIPPLRERVEDIPLLVEH
              *       *      * ** **  *       * ** *

                                                NBP
AcoR  Msitri  FRIEQEQLGVAEAHSFADEVWRVFLQHPWPGNIREARNVLRSSIAVARER
AcoR  Raleut  LLQEGSAEGSE--ITLSPAARERLHRHRWPGNLRELRNVLEYARAVCADG
DmpR  Pseput  FLQRFHQEYGKRTLGLSDKALEACLHYSWPGNIRELENVIERGIILTDPN
TbuT  Ralpic  FLQRMAKKHDKQITGFRERAVDALFAYDWPGNVRELENMIERAVILAEDG
HbpR  Pseaze  LIARFSKRHGRKLKGISSAAMQVLIYHDWPGNIRELENVIERAIIMAQDV
DctD  Rhileg  FAARAAERFRRDVPPLSPDVRRHLASHTWPGNVRELSHYAERVVLGVEGG
NifA  Sinmel  FLQRFNEENGR-DLHFAPSALDHLSKCKFPGNVRELENCVRRTATLARSK
PilR  Pseaer  ILKRLAGDTGLPAARLTGDAQEKLKNYRFPGNVRELENMLERAYTLCEDD
NtrC  Saltyp  FLQVAARELGVEAKLLHPETETALTRLAWPGNVRQLENTCRWLTVMAAGQ
FlbD  Caucre  FVKKYSAANGIEEKPISAEAKRRLIAHRWPGNVRELENAMHRAVLLSAGP
XylR  Pseput  FLRRHHKEYGKKTLGLSDRAMEACLHYQWPGNIRELENALERGVILTESN
                                                  *** *
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B

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AcoR  Msitri  ----------EHRLEESGETSLDLSDWEARAVRFALASSNGNIAKA
AcoR  Raleut  ----------PAQSPAAAPFDPHQLPPEGMLLMQYLRASGWNLSAV
DmpR  Pseput  ----------GSWISQLLSSG-LSLDEIEESLMREAMQQANQNVSGA
TbuT  Ralpic  ----------EDVASSTTAIPRPGLAETEVAMLRAAVIEANGNLSRA
HbpR  Pseaze  NAAENANPKVLSLDEFAEQMVHQGSINLDQVQDAITRAAVKHSGGNISRA
DctD  Rhileg  ------------------------ERLERYEAEIIRDTLSANDGDVRRT
NifA  Sinmel  ----------DVPPKEPGSAGVASNLIERDRLISALEEAGWNQAKA
PilR  Pseaer  ----------AASLSEIDNLEDYLEDIERKLIMQALEETRWNRTAA
NtrC  Saltyp  ----------ALRSGHQNLLSEAQPELERTLLTTALRHTQGHKQEA
FlbD  Caucre  ----------AASRAFVGSTVAEVEQQLIIDTLEHCLGNRTHA
XylR  Pseput  ----------SWFRQIIDQG-VSLEDLEAGLMRTAMDRCGQNISQA

                        Helix-Turn-Helix
AcoR  Msitri  ARSLGITRATLYHKMARYGLQSDRRIISKR------
AcoR  Raleut  ARQIGVSRMTLYRRMERYGIQSPNRRDGGPEPTDA-
DmpR  Pseput  ARLLGLSRPALAYRLKKIG----IEG----------
TbuT  Ralpic  ARVLGISRPTLAYRLRKYQIP--VDGS---------
HbpR  Pseaze  ASLLGITRARLDYRVKKIT----------------
DctD  Rhileg  IEALGIPRKTFYDKLQRHGINRGGYSSRK-------
NifA  Sinmel  ARILEKTPRQVGYALRRHGVDVRKL-----------
PilR  Pseaer  AQRLGLTFRSMRYRLKKLGID---------------
NtrC  Saltyp  ARLLGWGRNTLTRKLKELGME----------------
FlbD  Caucre  ANILGISIRTLRNKLKEYSDAGVQVPPPQGGVGAAA
XylR  Pseput  ARLLGLTRPAMAYRLKKLDPSLSVKAMGR-------
```

Figure 6.2 Amino acid alignments of central (A) and c-terminal domains (B) of an AcoR-like proetin form *M. trichosporium* OB3b with NifA-family regulators.
Alignments were generated using the ClustalX program. Conserved residues between all 10 regulators are indicated by stars below the alignment.
A, Walker A and B motifs are overlined by a thin black bar, the conformational switch motif is similarly indicated and the nucleotide binding sequence in Red. The conserved aspartate residue believed to be the site of phosphorylation is highlighted in bold type.
B, The putative helix-turn-helix DNA-binding domain is overlined in blue.
Key to organisms: Msitri, *Methylosinus trichosporium* OB3b; Raleut, *Ralstonia eutrophus*; Pseput, *Pseudomnas putida*; Ralpic, *Ralstonia pickettii*; Pseaze, *Pseudomonas azelaica*; Rhileg, *Rhizobium leguminosarum*; Sinmel, *Sinorhizobium meliloti*; Pseaer, *Pseudomonas aeruginosa*; Saltyp, *Salmonella typhimurium*; Caucre, *Caulobacter crescentus*. The functions of the activator proteins used in this alignment are listed in Table 6.1.

The AcoR-like protein from *Methylosinus trichosporium* OB3b is 649 amino acids in length with a predicted molecular weight of 7,330 kDa and possesses a long N-terminal domain, which is similar, but not homologous to, the AcoR protein from *Ralstonia eutrophus* H16. The N-terminal domains of proteins from this family of proteins are highly variable and it is proposed that they confer effector specificity to these proteins. However, the N-terminal region of the *acoR*-like gene has only low (~ 24 % identity at amino acid level) with several hypothetical transcriptional regulators from, *Bacillus subtilis, Pseudomonas stutzeri* and probable AcoR proteins from *Bacillus halodurans* and *Pseudomonas aeruginosa* (Identified using the BlastX tool at www.ncbi.nlm.nih.gov/blast).

Analysis of the sequence 5' of the *acoR*-like gene reveals the presence of a possible Shine-Dalgarno sequence (GGGA), but no putative promoters or regulatory

sequences have been identified. It may be that such elements are present further upstream from these genes than the sequenced region.

The open reading frames immediately 5' of *mmoX* encodes a member of the GroEL (Cpn60) protein chaperone family. It encodes a protein of 581 amino acids with a molecular mass of 62,231 Daltons and has 39% identity and 59% similarity with the GroEL protein from *Neisseria meningitidis* (Parkhill *et al.*, 2000). The protein sequence reveals the presence of a region of homology with the $F_1\alpha$ATPase subunits of ATP synthases and a valine proposed to be involved in GroEL oligomerisation (Tanaka *et al.*, 1997). Many GroEL proteins contain multiple Gly-Gly-Met (GGM) repeats at the extreme carboxy-terminal, which are absent from the GroEL from this organism (Tanaka *et al.*, 1997). It is worth noting that the predicted length of this protein is 581 amino acids, which is approximately 40 amino acids longer than most GroEL proteins. It possesses a putative Shine-Dalgarno sequence (GAGGA) 17 bp 5' of its start codon (Shine and Dalgarno, 1974) and a putative stable stem-loop structure (AAAGCGCTGCGGCGA-N_4-TTCGCCGCAGCGUUUU) (ΔG= -23.2kcals) 28 bp downstream of the 3' end of *groEL*. The presence of several U residues after the putative stem-loop indicates that this may be a *rho*-independent transcriptional terminator (Platt, 1981; Yanofsky, 1981) and that *groEL* is transcribed independently of the *mmo* operon. The absence of putative terminators between *acoR* and *groEL* indicated that they may be transcribed together. This theory was recently confirmed by Julie Scanlan who used RT-PCR to show that transcripts bridging the gap between *mmoR* and *groEL* were present in *Methylosinus trichosporium* OB3b grown in low-copper media. Therefore, in contrast to many *groEL* genes, the $groEL_{mmo}$ gene from *Methylosinus trichosporium* OB3b is not found in an operon with a *groES* gene. Its *groEL* gene lies in an operon with the *acoR*-homologue upstream of the *mmo* cluster, thus it will be referred to as $groEL_{mmo}$ from now on. Several bacteria contain individual *groEL* genes and always one or more complete *groESL* operon (Lund, 2001). The presence of a lone $groEL_{mmo}$ gene indicates that *M. trichosporium* OB3b may possess one or more *groESL* operons in its genome.

Many *groEL* genes are regulated in a negative manner by a repressor named HrcA, via binding to a 9 bp conserved inverted repeat sequence named a CIRCE element (Controlling Inverted Repeat of Chaperone Expression): TTAGCACTC-N_9-GAGTGCAA (Lund, 2001; Lemos *et al.*, 2001; Segal and Ron,

1996). Neither a CIRCE element nor putative promoter sequences are found upstream of *groEL*$_{mmo}$ or the *acoR*-like genes in *M. trichosporium* OB3b. Attempts to identify the transcriptional start site for the *acoR*-like and *groEL*$_{mmo}$ genes by primer extension were unsuccessful (data not shown). Recent evidence showing co-transcription of *mmoR* and *groEL*$_{mmo}$ explains why no primer extension products were gained for *groEL*$_{mmo}$, and it is possible that the origin of an *acoR-groEL*$_{mmo}$ transcript lies further upstream from the *acoR*-like gene than is contained in the cloned region. However, experiments performed subsequently by Julie Scanlan have shown that *acoR-groEL*$_{mmo}$ are transcribed in an *rpoN* mutant of *M. trichosporium* OB3b, showing that its transcription is σ^N-independent.

The presence of a member of the σ^N-dependent transcriptional activators in close proximity to the *mmo* cluster, which is known to be regulated in a σ^N-dependent fashion, indicated that it probably played an important role in transcription of the *mmo* operon. Several bacterial operons are regulated in a σ^N-dependent manner by a positive activator proximal to the cognate gene cluster, e.g. *touR, tbuT, acoR, xylR* (Arenghi *et al.*, 1999; Byrne & Olsen, 1996; Krüger and Steinbüchel, 1992; Inouye *et al.*, 1988) Thus an investigation of a knockout of the *acoR*-like gene was performed.

6.3 Construction of a vector for the insertional knockout of an *acoR*-like gene

In order to examine whether the *acoR*-like gene product played a role in regulation of transcription of the *mmo* operon, a 'knockout vector' using the narrow-host range vector pK18*mob* was constructed. Briefly, a 4,424 nucleotide *Hin*dIII-*Bam*H1 fragment containing the *acoR* and *groEL* homologues was ligated into pK18*mob* before deletion of a 1,014 bp *Sal*I fragment from *acoR* which was replaced by a Gm^R cassette from p34S-Gm to create pJS1 (Figure 6.3). The construction of plasmid pJS1 (and strain JS1) was performed by Julie Scanlan.

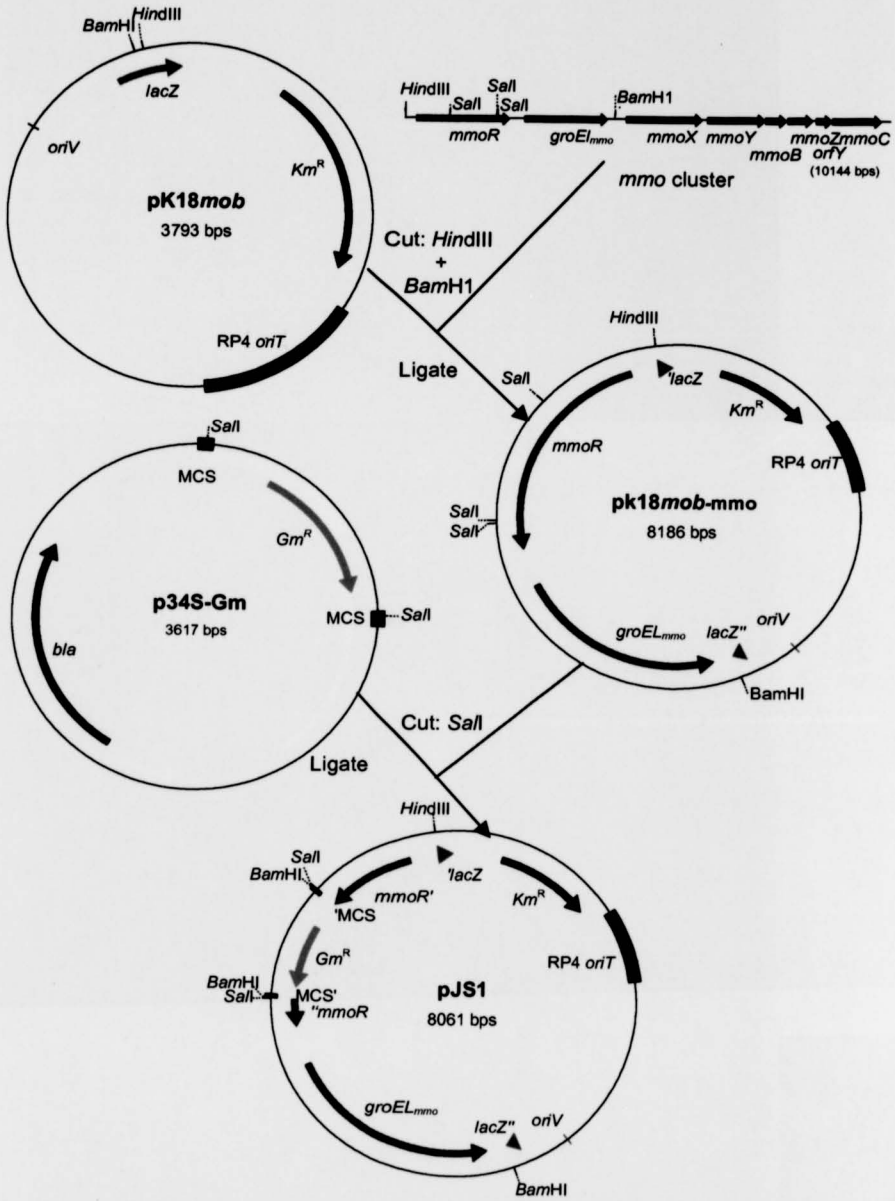


Figure 6.3 Cloning scheme for construction of pJS1. A 4,424 bp *Hin*dIII-*Bam*HI fragment containing *mmoR* and *groELmmo* was ligated into the *lacZ* gene of pK18*mob*. A 1,014 bp *Sal*I fragment was then deleted from the *acoR*-like gene and replaced by a Gm^R cassette from p34S-Gm to give pJS1.

6.4 Marker-exchange mutagenesis of an *acoR*-like gene

In order to inactivate the *acoR*-like gene from *Methylosinus trichosporium* OB3b, the plasmid pJS1 was transferred into *E. coli* S17-1 before filter mating with *M. trichosporium* OB3b. Exconjugants were selected on NMS agar plates containing gentamycin (Gm). By this method 300 Gm resistant colonies were obtained. After picking onto plates containing kanamycin (10 μg/ml) and Gm (5 μg/ml), 8 of these strains were found to have a Gm^RKm^S phenotype. This indicated that a double crossover event resulting in the loss of the plasmid backbone, containing the kanamycin resistance gene, from the chromosome had occured.

One of these 8 strains, strain JS1, was selected for further study. Southern blotting revealed that this strain possessed a Gm cassette within its chromosome with a banding pattern predicted by a double crossover event when probed with the Gm cassette and an *mmoR* probe (data not shown)(J. Scanlan, personal communication).

The insertion of an antibiotic cassette in the *acoR*-like gene could have caused a polar mutation in the $groEL_{mmo}$ gene. However, the pJS1 construct contains a Gm^R cassette which lacks a terminator 3' of the Gm^R cassette. Therefore it was likely that this insertion would not be polar. RT-PCR experiments confirmed that *groEL*-specific transcripts are present in strain JS1, confirming a non-polar insertion in *mmoR* (J. Scanlan, unpublished). Thus, any phenotypes observed for the JS1 mutant would be due to a mutation in the *acoR*-like gene and not a polar effect on $groEL_{mmo}$.

6.5 Phenotypic characterisation of an *acoR*-like gene: renaming as *mmoR*

Chapter 5 described the construction and phenotypic characterisation of strain Gm1, containing an inactive *rpoN* gene, and revealed that the σ^N-sigma factor was involved in transcriptional initiation of the *mmo* operon and in several facets of nitrogen metabolism. Thus, the effects of a mutation in the *acoR*-like gene were analysed in a similar manner.

Firstly, strain JS1 is capable of utilising nitrate, ammonia, glutamine and dinitrogen as sole nitrogen sources (data not shown). Therefore, it is clear that the *acoR*-like gene product is not involved in the regulation of any aspects of nitrogen metabolism.

However, an insertion in the *acoR*-like gene does have a striking effect on the soluble methane monooxygenase gene cluster (*mmo*). The ability of strain JS1 to oxidise naphthalene to naphthol, an indicator of soluble methane monooxygenase enzyme activity, was tested from cultures grown on low-copper NMS agar plates and in low copper NMS liquid culture. These naphthalene assays showed that strain JS1 does not possess an active sMMO enzyme (Figure 6.4).

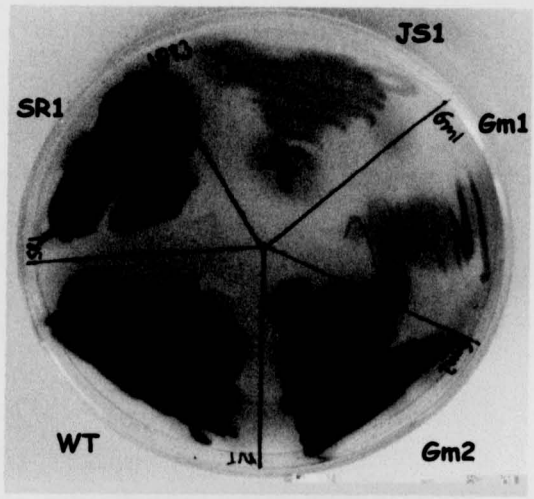


Figure 6.4 sMMO activity of Wild-type and JS1 strains of *Methylosinus trichosporium* OB3b. *M. trichosporium* OB3b strains WT, JS1, Gm1, SR1 and Gm2 were grown for 10 days on MS-Glutamine Noble agar plates containing no-added copper. sMMO activity was tested using the naphthalene assay developed by Brusseau *et al.*, (1990). After incubation of plates with naphthalene (30 min, 30°C) , oxidation to naphthol was tested by flooding the plates with tetrazotized *o*-dianisidine, which turns purple in the presence of naphthol, and stays orange in its absence. Strains SR1 and Gm2 are single recombinant *acoR* and *rpoN* strains containing active sMMO. Strain Gm1 is described in chapter 5.

To determine whether this loss of activity was due to the presence of an inactive enzyme or to the complete absence of its constituent subunits, SDS gels and Western blots using sMMO-hydroxylase-specific antibody were performed. Crude cell-free extracts were prepared from 50 ml cultures of both wild-type and JS1 mutants grown in low-copper NMS media. Approximately 90 µg of these extracts was loaded per lane on a 12% SDS PAGE gel (Figure 6.5). The 3 sMMO subunits are clearly visible in the wild-type extracts, but are absent from the JS1 (and Gm1) strain. A Western blot of this gel was also prepared and challenged with sMMO-hydroxylase-specific antibody. Although the antibody reacted non-specifically with the large subunit of methanol dehydrogenase, it was possible to

establish that the alpha and gamma subunits of the hydroxylase were absent from the JS1 (and Gm1) strains (Figure 6.5). These results were identical to those obtained for the Gm1 mutant and a discussion of the significance of this result can be found in section 5.6.3.

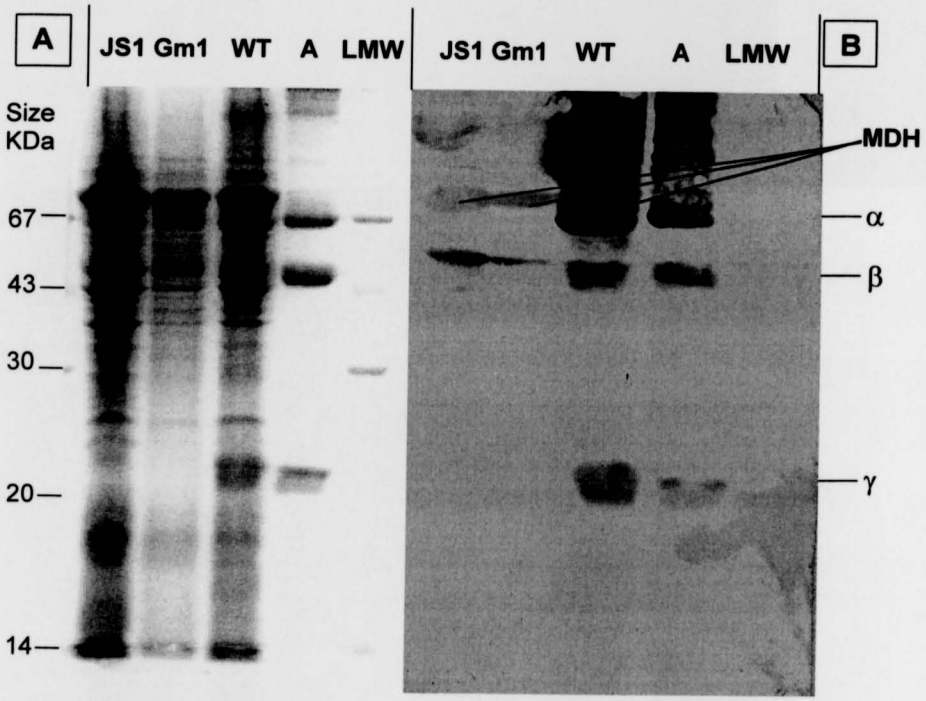


Fig 6.5 SDS PAGE and Western blot of *mmoR* and *rpoN* mutants Gm1 and JS1.
Whole cell protein was extracted (see section 2.20.2) from *M. trichosporium* OB3b Gm1, JS1 and wild-type (WT) strains grown on low copper MS medium containing 0.05 % Glutamine (w/v). Protein concentrations were estimated using the BioRad assay. Approximately 90μg of the total cell protein was loaded per lane on a 12% SDS-PAGE gel. The left hand half was stained with Coomassie Brilliant Blue (panel A) and the right hand half left unstained. This was electroblotted onto nitrocellulose membrane and challenged with sMMO-hydroxylase specific rabbit IgG (panel B). sMMO hydroxylase (Protein A) was a gift from Sue Slade. Size standards were Low Molecular Weight Markers (LMW) from Amersham Pharmacia. The α, β, γ subunits of the sMMO hydroxylase and the large subunit of methanol dehydrogenase (MDH) are labelled.

In addition to testing the presence of the sMMO-hydroxylase protein subunits, the presence of *mmoX* (encoding the alpha subunit of the sMMO

hydroxylase) transcripts was tested. Total RNA was extracted from cultures of wild-type and JS1 strains grown in low-copper NMS media. RT-PCR using the 206F and 886R primers (See section 4.2) was conducted and showed that there were no *mmoX*-specific transcripts in RNA extracted from the JS1 strain (figure 6.6).

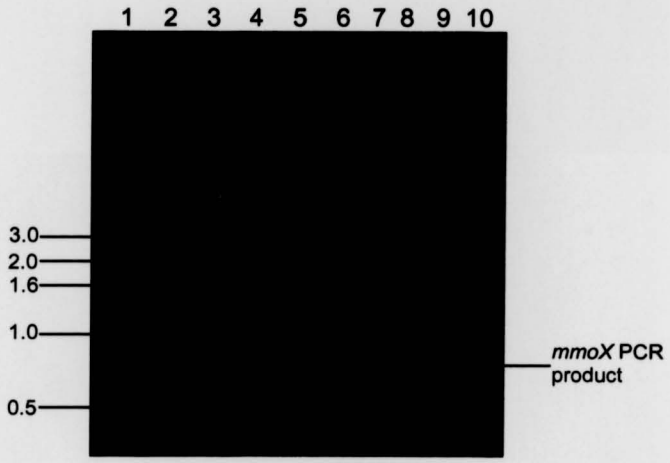


Figure 6.6 RT-PCR showing absence of *mmoX*-transcripts of strains JS1 and Gm1
RT-PCR was performed on total RNA extracted from the wild-type, JS1 and Gm1 strains grown on MS medium containing 0.05 % (w/v) glutamine as described in section 2.7. Primers used were 206F:ATCGCBAARGAATAYGCSCG and 886R:ACCCANGGCTCGACYTTGAA; Products were analysed on a 2% TBE agarose gel. Lanes: 1, 1kb ladder; 2, WT cDNA; 3, WT RNA; 4, JS1 cDNA; 5, JS1 RNA; 6, Gm1 cDNA; 7, Gm1 RNA; 8, DNA +ve; 9, -ve; 10, 1kb. All RNA preparations were tested for contaminating DNA using *mmoX* PCR primers and were negative in all cases. The RNA used in lanes 3, 5 and 7 originated from independent cultures and this result was confirmed using a further 2 independent cultures.

These results clearly show that the *acoR*-like gene from *Methylosinus trichosporium* OB3b is indispensable for the regulation of the *mmo* operon and that it acts at the level of transcription. Due to its important role in the regulation of the *mmo* operon, the renaming of the *acoR*-like gene as *mmoR* (methane monooxygenase Regulator) is proposed.

6.6 Discussion

Sequencing of the region upstream of the *mmo* operon of OB3b revealed the presence of two previously unidentified open-reading frames, *mmoR* and $groEL_{mmo}$ (*cpn60*). The first of these is a single *groEL* gene with similarity to the *groEL* gene from *Neisseria meningitidis* and many other bacteria. The presence of the $groEL_{mmo}$ gene in an operon with the *mmoR* gene is unusual, and probably unique, in that *groEL* genes are found in an operon with a *groES* gene or as individual *groEL* genes (Lund, 2001). Single *groEL* genes are often found in bacteria with multiple *groEL* genes such as *Mycobacterium leprae* (Rinke de Wit *et al.*, 1992), *Sinorhizobium meliloti* (Rusanganwa and Gupta, 1993), *Rhodobacter sphaeroides* (Lee *et al.*, 1997) and *Synechococcus vulcanus* (Tanaka *et al.*, 1997). Therefore, it is likely that the presence of a lone $groEL_{mmo}$ gene in *Methylosinus trichosporium* OB3b suggests that it contains a complete *groEL* operon elsewhere in its genome. Indeed, a preliminary scan of the unfinished genome of the methanotroph *Methylococcus capsulatus* Bath reveals the presence of two *groESL* operons, both possessing CIRCE elements with putative σ^{70} promoters, and one lone *groEL* gene (http://tigrblast.tigr.org/ufmg/).

Multiple *groEL* genes are not always all expressed, for example a transcript for the $groESL_2$ operon of *Rhodobacter sphaeroides* is not detectable under heat-shock or normal growth conditions (Lee *et al.*, 1997). In contrast the five *groESL* operons from *Bradyrhizobium japonicum* are all expressed to different degrees under certain conditions (Fischer *et al.*, 1993). In fact one of these operons, $groESL_3$ is co-regulated with the nitrogenase genes in a mechanism dependent on NifA and σ^N (Fischer *et al.*, 1993). Recent RT-PCR experiments have shown that $groEL_{mmo}$ is transcribed under low-copper conditions in a σ^N-independent manner with the *mmoR* gene (J Scanlan, unpublished results). The absence of recognisable regulatory sequence motifs from the region 5' of $groEL_{mmo}$ and its co-transcription with *mmoR* suggests that both the genetic location and regulation of this gene may be unique. An investigation of $groEL_{mmo}$ transcription by RT-PCR and Northern blotting in response to copper should yield interesting information regarding this discovery.

The finding that $groEL_{mmo}$ is co-transcribed with *mmoR* raises the possibility that $groEL_{mmo}$ gene is involved in the assembly of the sMMO enzyme. In most cases the GroEL protein is believed to be relatively promiscuous in the range of proteins on which it assists folding. But it is becoming clear that in some cases specific GroEL

proteins may act on specific sets of proteins (Lund *et al.*, 2001). In *Sinorhizobium meliloti*, interruption of one of the *groEL* genes by Tn5 results in a mutant incapable of nitrogen fixation (reviewed in Lund *et al.*, 2001). A double knockout of two *groEL* operons from *Bradyrhizobium japonicum*, one of which is co-regulated with the *nif* genes, resulted in the loss of nitrogenase protein (Fischer *et al.*, 1999). It is tempting to speculate that the presence of a single *groEL* gene in the newly-extended *mmo* operon indicates that it may play a role in folding of a functional sMMO-complex. This speculation is further fuelled by the presence of a lone *groEL* gene located just 198 bp 3' of *mmoC*, the final gene of the sMMO operon of *Methylococcus capsulatus* Bath. Its presence so close to the *mmo* operon may be merely coincidental, but the absence of putative terminators in the region between *mmoC* and *groEL* make it noteworthy. The construction of an insertional mutant of the $groEL_{mmo}$ gene from both *M. trichosporium* OB3b and *Methylococcus capsulatus* Bath would reveal whether it is specialised in assisting the folding of the sMMO enzyme complex, and is an experiment currently underway in the Murrell laboratory.

The furthest 5' of these open reading frames is a gene, *mmoR*, which has strong homology with members of the σ^N (σ^{54}) dependent enhancer binding proteins (EBPs). The location of such a regulatory gene in close proximity to the gene cluster on which it acts is not unusual. Indeed, the *acoR* genes of *Clostridium magnum*, *Bacillus subtilis* and *Ralstonia eutrophus* all lie directly upstream from their respective acetoin catabolism gene clusters (Huang *et al.*, 1999; Krüger *et al.*, 1994; Krüger and Steinbuchel, 1992). However, the presence of the $groEL_{mmo}$ gene in this region is unusual and increases the suspicion that it may be an sMMO-specific chaperone.

Mutation of the *mmoR* gene by the insertion of an antibiotic cassette results in a strain unable to express the soluble methane monooxygenase gene operon. Sequence analysis suggests that *mmoR* is a member of a the EBP family of transcriptional regulators, which includes NifA, NtrC and AcoR. The EBPs exert transcriptional control from distant (usually 100- 200 bp) palindromic Upstream Activator Sequences (UAS) and activate transcription of genes transcribed by RNA polymerase holoenzyme containing the σ^N-subunit (Morrett and Segovia, 1993). EBPs are involved in the activation of a wide range of physiological processes including nitrogen fixation, nitrate assimilation, dicarboxylic acid transport, hydrogen

oxidation, toluene oxidation and *o*-xylene oxidation (Reviewed in Morrett and Segovia, 1993; Merrick, 1993). The central and C-terminal domain of the EBPs is highly conserved and is characterised by the presence of ATPase, Nucleotide binding and switch motifs within the central domain and a helix-turn-helix DNA-binding motif at the C-terminal end. The N-terminal region of these proteins is variable both in sequence and length. However, those members of the EBPs which are part of a two-component sensor-activator pair display a high degree of homology, e.g. NtrC from *E. coli* and *dctD* from *Sinorhizobium meliloti*. The N-terminal domains of NifA proteins also share homology in a region believed to be involved in interaction with its co-regulator NifL (Morrett and Segovia, 1993). The *mmoR* gene, like *acoR* from *Ralstonia eutrophus* H16 (Kruger *et al.*, 1992) and TouR from *Pseudomonas stutzeri* (Arenghi *et al.*, 1999), has an extended N-terminal region. These extended N-terminal regions contain the sensory domains. In the case of AcoR and TouR, they are believed to bind the aromatic compounds over whose metabolism they exert control (Krüger and Steinbüchel, 1993; Arenghi *et al.*, 1999). In fact, a recent study by Garmendia *et al.*, (2001) succeeded in shuffling the N-terminal sensory domain of XylR from *Pseudomonas putida* with the corresponding domains from other regulators to expand the range of aromatic compounds to which it was able to respond. It therefore seems likely that the extended N-terminal region of *mmoR* also serves such a function in signal sensing and transduction.

The MmoR protein exerts positive regulation over the *mmo* operon under conditions of copper starvation. Thus it is possible that the MmoR protein is in some way modified in response to low copper conditions. The simplest situation would exist whereby the MmoR protein is inactivated by the binding of copper under copper-sufficient conditions whilst being active in the absence of copper. An analysis of the N-terminal region does not reveal typical copper binding motifs, such as the multiple Cys-X-X-Cys motifs observed in the copper metallochaperones or copper ATPase proteins of both eukaryotes and prokaryotes (Koch *et al.*, 1997; Strausak *et al.*, 1999)(See section 1.10). To the best of my knowledge, the only example of a metal-regulated EBP is ZiaR of *E. coli*, which is believed to play a role in the homeostasis of zinc ions. However, this is a member of a two-component pair comprising of ZiaR and ZiaS, in which ZiaS is the sensor of Zinc (Reitzer & Schneider, 2001). Neither does *mmoR* contain the four conserved cysteine residues believed to bind a metal ion in the region between the central and C-terminal domains

of oxygen sensitive NifA proteins from several bacteria (Morrett and Segovia, 1993). Thus it is not clear how MmoR senses copper ions or indeed if it binds copper ions at all.

As discussed above, the N-terminal regions of the NifA EBPs share homology in a region which allows interaction with NifL. This protein inhibits the action of NifA by direct protein-protein contact, which prevents the interaction of NifA with the σ^N protein (Lei *et al.,* 1999, Dixon, 1998)(See section 1.9). The activity of the PspF protein of *E.coli* is also mediated by direct protein-protein interaction. In this case, PspF lacks an N-terminal domain and is constitutively active *in vivo* and *in vitro*. The PspA protein is believed to inhibit PspF by binding to the central domain (Dworkin *et al.,* 2000). Thus it is possible that the *mmoR* protein has constitutive activity and its function is modulated by the binding of a protein expressed in the presence of copper.

The data presented in this chapter adds to that from chapter 5 and confirm that the *mmo* operon of OB3b is transcribed in response to low copper levels from a σ^N-promoter in a σ^N- and *mmoR*-dependent manner. The identification of a transcriptional regulator for the *mmo* operon is a major breakthrough in the study of methane monooxygenase gene regulation and opens many avenues for further study. As discussed in section 5.6.3, the presence of σ^N -type promoters 5' of *mmoX* genes from several methanotrophs raises the possibility of a widespread mode of regulation for *mmo* operons. Further sequence information for the regions surrounding these gene clusters may reveal the presence of *mmoR*-like genes. As mentioned previously analysis of the region downstream of the *mmo* operon of *Methylococcus capsulatus* Bath reveals a *groEL* gene 3' of *mmoC*. It also revealed a two-component sensor-regulator pair and an EBP encoding gene which is believed to be involved in the regulation of this *mmo* operon (R. Csaki, Personal communication).

At this early stage, the mechanism of how MmoR senses copper remains an enigma. Does it directly bind copper? Is it regulated by protein-protein interaction? Is there an unknown inducer molecule? Is MmoR itself regulated in response to copper? It is clear that in order to find any of the answers to these questions the MmoR protein must initially be expressed in *E.coli* and purified in order to study its DNA binding properties, particularly in response to copper ions. Knowledge of the conditions under which *mmoR* is transcribed would also yield information regarding how this

unique copper regulated system is controlled. The data presented in chapters 5 and 6 may provide the first example of an EBP which is directly responsive to a metal ion.

In *Methylosinus trichosporium* OB3b the *mmo* operon is transcribed under low copper-to-biomass ratios whereas when copper levels increase *mmo* transcription is abolished and *pmo* transcription induced (Nielsen *et al.*, 1997). However, discovery that *rpoN* and *mmoR* are involved in the regulation of the *mmo* operon still leaves us with no knowledge of the factors controlling the transcription of the *pmo* operon. Recent findings regarding the *pmo* operon indicate that it is transcribed from a σ^{70}-promoter (Chapter 3), and is not controlled by σ^{N} or *mmoR* since strains Gm1 and JS1 can still use methane as a carbon source. The switch between *mmo* and *pmo* expression in response to copper must involve a system which senses copper levels and transmits this signal to *mmoR* and an unknown transcription regulator of the *pmo* operon. This would result in the deactivation of *mmo* transcription and up-regulation of *pmo* transcription, possibly by a derepression mechanism. In addition, if *mmoR* directly binds copper ions, then it is possible that a metallochaperone (Harrison *et al.*, 2000; Cobine *et al.*, 1999) delivers this copper to *mmoR* since cytoplasmic free-copper levels are believed to be almost zero (Lippard, 1999). In short, there are likely to be several other gene products involved in the regulation of methane monooxygenase genes and it is the search for such factors which forms the basis for Chapter 7.

Chapter 7

The search for new genes involved in the copper switch

7.1 Introduction

The 'copper switch' is the term used to describe the unique copper-responsive regulatory switch controlling expression of the soluble and particulate methane monooxygenase gene clusters in methanotrophic bacteria (Stanley *et al.*, 1983). Stanley *et al.*, (1983) reported the existence of two forms of methane monooxygenase, with the membrane-bound (particulate) form being expressed at high copper to biomass ratios, and the soluble form at low copper to biomass ratios. Since then, the genes encoding the structural peptides for both the particulate and soluble methane monooxygenase enzymes have been cloned from *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991; Gilbert *et al.*, 2000), *Methylococcus capsulatus* Bath (Stainthorpe *et al.*, 1990; Stolyar *et al.*, 1999) and *Methylocystis* sp. strain M (McDonald *et al.*, 1997; Gilbert *et al.*, 2000).

In chapters 5 and 6 of this thesis the cloning and sequencing of two new operons from *Methylosinus trichosporium* OB3b: the *rpoN* and *mmoR-groEL*$_{mmo}$ operons, are reported and were shown to be involved in transcriptional regulation of the sMMO operon. However, the regulation of the *pmo* and *mmo* operons is complex, and in addition to the changes in their expression levels, copper must affect expression of other genes. In common with several other organisms it would be expected that *Methylosinus trichosporium* OB3b possesses a copper homeostasis system which is capable of sensing extracellular levels of copper, allowing it to take in or expel copper as required. Indeed, the first insights into copper transport in methanotrophs were reported by Dispirito *et al.*, (1998) and Tellez *et al.*, 1998) who isolated small octapeptide copper-binding compounds (CBCs) from the spent culture medium of both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath.

In addition to high copper levels causing 'up-regulation' of *pmo* expression it is also clear that an extensive intracellular membrane network is constructed (Scott *et al*, 1981; Stanley *et al.*, 1983) (Section 1.5.1). These easily observable changes to the cell in response to copper levels represent a suite of genes, which are regulated in response to copper ions and should be identified in order that we understand this unique phenomenon more fully. It is the search for such new components of what is likely to be a copper-responsive regulon, which forms the basis of this chapter.

7.2 Tn5 Transposon mutagenesis

7.2.1 Rationale and experimental plan

The aim of the experiments presented in this chapter were to identify new genes, the products of which were directly involved in the regulation of the methane monooxygenase genes or indirectly involved in processes such as copper transport. The first technique employed was transposon mutagenesis. This relies on the random insertion of a transposable element, a transposon, into the chromosome of the organism of interest, the presence of which can be detected by the action of its associated antibiotic resistance gene. A transposon insertion causes a mutation of the gene into which it transposes and provided an adequate phenotypic selection procedure is available, allows the identification of genes involved in a particular process.

The general structure of a transposon comprises of two insertion sequences (IS) which facilitate the random insertion of the DNA contained between the two IS elements (Berg and Berg, 1983). Between these IS elements, there typically resides a transposase gene, encoding the enzyme responsible for insertion of the transposon into the chromosome of the target strain. In the case of Tn5, an inhibitor is encoded in the transposase region, preventing multiple insertions of the transposon into the chromosome (Berg and Berg, 1983; DeLorenzo *et al.*, 1990).

The mini-Tn5 transposon and plasposon-type transposons were chosen for use in these experiments for several reasons. Firstly, Tn5 has been extensively used for generating random mutations in many Gram-negative bacteria including genera such as *Bordetella, Salmonella, Proteus, Vibrio, Rhizobium, Alcaligenes* and *Caulobacter* (DeLorenzo *et al.*, 1994; Berg and Berg, 1983, Herrero *et al.*, 1990). Secondly, several narrow-host range Tn5 delivery suicide vectors, containing the RP4 mobilisation element, allowing transfer from *E. coli* to *Methylosinus trichosporium* OB3b are available. Thirdly, the mini-transposon and plasposon vectors have been engineered to enhance their use in generating random mutations (DeLorenzo *et al.*, 1990; Dennis and Zylstra, 1998).

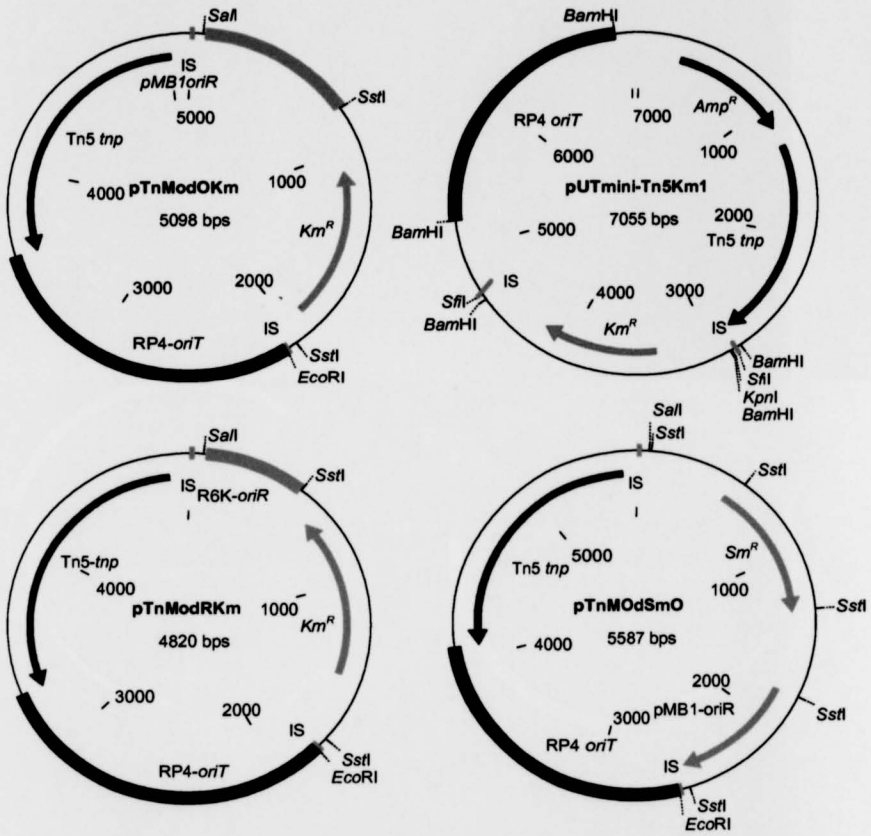


Figure 7.1 Plasposon and minitransposon Tn5 delivery vectors used in this study.
Construction of pTnModSmO, pTnModOKm and pTnModRKm (plasposons) is described in Dennis and Zylstra, (1998). pUTminiTn5Km1 (minitransposon) is described in DeLorenzo *et al.*, (1990).

Both the Mini-Tn5 and plasposon transposable elements contain the transposase gene on the suicide vector but outside the IS elements. In this way it is able to facilitate insertion of the Tn5 element into the chromosome, whilst the transposase is lost from the cell upon loss of the suicide vector backbone. This should ensure that only one transposon insertion occurs per cell. Another advantage of these elements is that they no longer contain the transposase inhibitor gene and so it is possible to introduce additional Tn5 elements into a mutant of interest at a later date. The Mini-Tn5 vector employed in this study was pUTmini-Tn5Km1 constructed by

De Lorenzo *et al.,* (1990) (Figure 7.1). In addition, the plasposon vectors pTnModSmO, pTnModOKm and pTnModRKm, were also used (Dennis and Zylstra, 1998) (Figure 7.1). The plasposons differ from the the MiniTn5 construct by virtue of the fact that the origin of replication for the plasmid lies within the transposable region. This allows easy cloning of the Tn5 insertion element and flanking DNA by digestion of the chromosomal DNA of a transposon mutant of interest, followed by recircularisation of the fragments and transformation into the appropriate strain of *E.coli*. This will result in a plasmid which contains the flanking DNA of interest, which can then be sequenced.

The use of RP4*mob*-containing suicide vectors for the inactivation of specific genes in *Methylosinus trichosporium* OB3b has been demonstrated by Martin and Murrell (1995) and in this thesis (see also section 1.6)(Chapters 5 and 6). Thus, it should be possible to introduce these transposon-containing suicide vectors into *Methylosinus trichosporium* OB3b by conjugation as described in section 2.9. The transposon containing transconjugants could then be selected on the basis of their antibiotic resistance profiles and the transposon clone library screened for phenotypes.

Initially, a method was devised to screen a library of mutants for their ability to utilise the sMMO-specific substrate naphthalene using the plate assay described in section 2.23. In this way, it should have been possible to isolate not only mutants in the structural genes of the sMMO cluster, but more interestingly, transcriptional regulators of the sMMO operon. The other possible cause for a sMMO$^-$ phenotype would be an insertion in a copper transport or copper sensing gene. All of which would provide new information regarding sMMO regulation and copper homeostasis in this organism. Screening for pMMO defective phenotypes may have proved more difficult, but by producing libraries of transposon mutants selected under low copper conditions (where a pMMO mutation would be silent) should have allowed pMMO$^-$ mutants to be isolated by an inability to grow on high copper agar medium. Also, since there are two copies of the *pmo* gene cluster in *Methylosinus trichosporium* OB3b (Gilbert *et al,* 2000) and in theory only one gene should be interrupted per cell, the chances of obtaining a mutation in a pMMO specific regulator might be increased.

7.2.2 Attempts to construct transposon libraries of *Methylosinus trichosporium* OB3b

The vectors shown in table 7.1 were maintained in *E.coli* S17-1 for Km resistant plasmids, and in *E.coli* JM109 for pTnModSmO. Filter-matings were performed as described in section 2.6. For plasmids in *E.coli* S17-1 bi-parental matings between donor strain and *Methylosinus trichosporium* OB3b were performed. However, in the case of pTnModSmO a tri-parental mating involving the helper plasmid, pRK2013, in *E.coli* strain HB101; *E.*coli JM109 containing pTnModSmO and *Methylosinus trichosporium* OB3b was carried out. Conjugation utilising the helper plasmid pRK2013 was first described by Figurski and Hellinski (1979) and allows high efficiency transfer of plasmids containing the RP4 determinant between strains.

Table 7.1 Vectors used in the attempted construction of transposon libraries in *Methylosinus trichosporium* OB3b. Descriptions of the plasmids can be found in table 2.3

Vector	Transposition frequency	Spontaneous resistance
pTnModSmO	$1 \times 10^{-7} - 1 \times 10^{-9}$	$1 \times 10^{-7} - 1 \times 10^{-9}$
pTnModOKm	$< 10^{-10}$	$< 10^{-10}$
pTnModRKm	$< 10^{-10}$	$< 10^{-10}$
pUTminiTn5Km	$< 10^{-10}$	$< 10^{-10}$
pJB3Km1	1×10^{-7}	$< 10^{-10}$

After mating, the mixtures were picked onto NMS agar containing Sm (20 μg/ml) or Km (10μg/ml) and a copper concentration of approximately 4 μM, which should allow sMMO defective mutants to grow, since pMMO would be expressed under these conditions. However, as the data in Table 7.1 illustrate, the production of transposon libraries using pTnModOKm, pTnModRKm or pUT-Tn5Km was unsuccessful. In fact transconjugants were never gained using these transposon vectors. It was possible that this lack of transconjugants could have been due to the kanamycin resistance determinant not functioning in these organisms. However, a control conjugation using the broad host range plasmid pJB3Km1 (Blatny *et al.,* 1997) illustrated that transfer of plasmids containing this kanamycin resistance determinant was possible in *M. trichosporium* OB3b, albeit at a low frequency (1×10^{-8}).

In contrast, it was possible to isolate putative transconjugants using the pTnModSmO transposon delivery system at a frequency of $1x\ 10^{-7}$ to 1×10^{-9}.

Unfortunately this is in the same order of magnitude as the frequency of spontaneous antibiotic resistance observed with streptomycin and may have therefore represented only spontaneous antibiotic resistance. Attempts to use streptomycin and spectinomycin (10 mg/ml), (to which pTnModSmO also encodes resistance) resulted in no putative transconjugant colonies being obtained.

7.2.3 Analysis of Sm-resistant *Methylosinus trichosporium* OB3b clones

In order to test whether the Sm resistance exhibited by these clones was due to spontaneous antibiotic resistance or from the introduction of the Sm resistance determinant from pTnModSmO into these clones, colony blots of 30 Sm^R clones was performed using the Sm^R cassette from pTnModSmO as a probe. Of the 30 (from a total of 1,000) putative transconjugants tested, all contained the Sm^R cassette originating from pTnModSmO (Data not shown), suggesting that the transposon had inserted into the chromosome of *Methylosinus trichosporium* OB3b.

In order for such a library to be of use in the identification of novel genes involved with causing $sMMO^-$/ $pMMO^-$ phenotypes it must contain a suite of single random insertions in its chromosome. Thus, DNA was extracted from ten of these putative transconjugants and digested with *Bam*HI. This enzyme does not cut within pTnModSmO, so any hybridising fragments would arise from restriction at sites flanking an insertion of the transposable element. Therefore, random insertion of the transposon (containing the Sm^R gene) should result in each clone containing one fragment, of a different size in each of the ten clones which hybridises to the Sm^R cassette. However, Figure 7.2 shows that there are 4 rows of hybridising bands of the same size in all 10 clones. This suggests that whilst containing the Sm^R cassette and therefore the transposon, its insertion has not occurred in a random manner, and has possibly inserted into the chromosome in multiple copies.

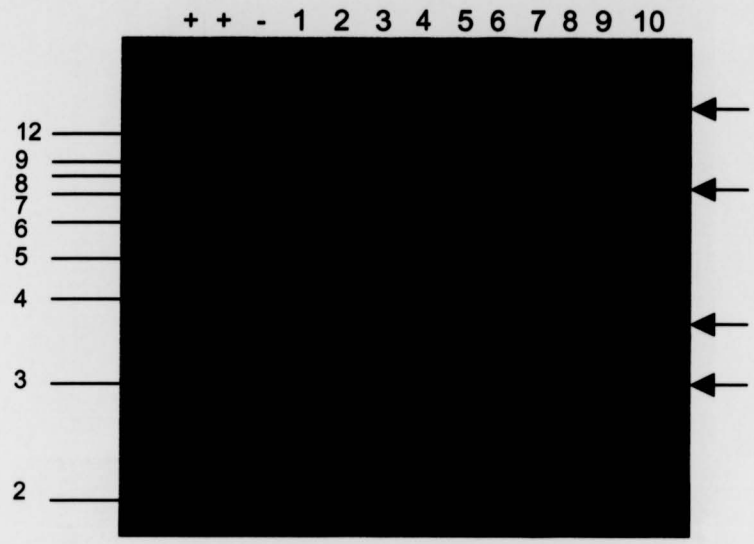


Figure 7.2 Southern blot of pTnMosSmO generated streptomycin resistant strains of *M. trichosporium*OB3b probed with Sm^R cassette from pTnModSmO.
Chromosomal DNA was extracted from 10 Sm^R strains of *M. trichosporium* OB3b (labelled 1-10) and the Sm^S wild-type strain (labelled -). All 10 Sm^R srains and the wild-type were digested with *Bam*HI. Two positive controls using undigested and *Sst*I digested pTnModSmO plasmid DNA were also included. The probe was excised from pTnModSmO plasmid DNA using *Sst*I. Size markers (kb) are shown and the four rows of hybridising bands are indicated by arrows. The blot was hybridised at 65°C overnight and washed at 65°C in 1 x SSC.

The phenomenon of insertion of Tn5 into 'hot-spots' is described by Berg and Berg, (1983), whom observed that Tn5 inserted into the plasmid pBR322 at sites scattered around its length, but more frequently at two specific sites within the Tet^R gene. However, these sites did not show a consensus sequence and so the rules governing such events were not established fully. In light of this information, the fact that the screening of 1,000 putative pTnModSmO generated clones did not reveal any $sMMO^-$ phenotypes (using the naphthalene assay) was not surprising.

These experiments revealed that Tn5 from pTnModSmO appears to insert into the chromosome of *Methylosinus trichosporium* at specific 'hot-spots' in its chromosome and that even this event occurred at a low frequency ($1 \times 10^{-7} - 1 \times 10^{-9}$), not significantly higher than the spontaneous rate of Streptomycin resistance. Therefore, although this method remains both an elegant and practical method for identifying genes involved in a specific process, it is, at this time, not a viable option for *Methylosinus trichosporium* OB3b.

7.3 Attempts to identify homologues of copper transport genes in *Methylosinus trichosporium* OB3b

Copper is an important trace element for biological processes in all known life forms, including humans. However, the properties that have made copper essential also make it a potent cytotoxin when homeostatic controls fail (Mercer, 2001). Thus, highly specialised copper transport systems have evolved in both eukaryotes and prokaryotes (Cooksey, 1993; Koch *et al.*, 1997; Harrison *et al.*, 2000). It is therefore likely that *Methylosinus trichosporium* OB3b also contains specialised copper homeostasis mechanisms. However, little is known about such systems in methanotrophs. Thus, the aim of this section was to try to identify copper transport components in *Methylosinus trichosporium* OB3b by heterologous probing.

7.3.1 Heterologous probing of the chromosome of *Methylosinus trichosporium* OB3b with the genes of the *copYZAB* operon from *Enterococcus hirae*

One of the best characterised prokaryotic copper homeostasis systems is the *copYZAB* operon from the Gram-positive enteric pathogen *Enterococccus hirae*. The *copA* and *copB* genes code for copper uptake and efflux proteins, CopA and CopB respectively (Odermatt *et al.*, 1993), which are members of a large group of ATP-dependent cation transporters (Solioz and Vulpe, 1996). In addition the operon encodes a transcriptional regulator, CopY and a metallochaperone, CopZ, which delivers copper (I) ions to the CopY protein (Odermatt and Solioz, 1995; Cobine *et al.*, 1999; Strausak and Solioz, 1997). The genes of this operon were used as heterologous probes to try to identify similar systems in *M. trichosporium* OB3b.

DNA was extracted from *Enterococcus hirae* as described in section 2.5.3 and PCR primers specific for each gene were used to amplify the individual genes of the operon (Figure 7.3)(Primers are listed in Table 7.2). The gene specific PCR products were cloned using a TOPO-TA kit from Invitrogen into the vector pCR2.1-TOPO and each clone was sequenced in order to verify its identity. These clones were then used as templates for the production of PCR-derived *cop* gene specific heterologous probes.

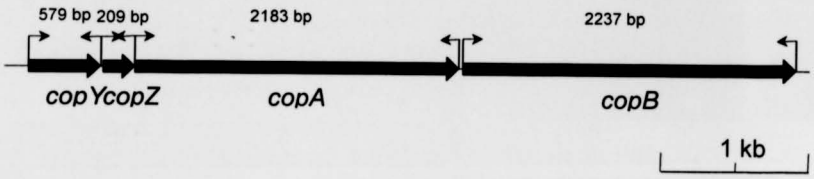


Figure 7.3 Genetic organisation of *copYZAB* operon from *Enterococcus hirae*.
The location of primers and sizes of PCR products are shown. Primer sequences are shown in table 7.2

Table 7.2 Primers used in the amplification of the *cop* genes from *Enterococcus hirae*.
All PCR reactions were performed with an annealing temperature of 50°C.

Gene target	Sequence	Function	Reference
CopA: CopA1	ATGGCAACGAATACCAAAAT	Copper influx-ATPase	Odermatt *et al.*, (1993)
CopA2	TTTGATCGTTTTTCGATTTA		
CopB: CopB1	ATGAATAATCGAATAGATCC	Copper efflux-ATPase	Odermatt *et al.*, (1993)
CopB2	TTTTAAAGTTAAGGCGTTGA		
Copy: CopY1	ATGGAAGAAAAGAGAGTATT	Transcription factor	Odermatt and Solioz (1995)
CopY2	TTACTGTTTCTTACATTTCAC		
CopZ: CopZ1	ATGAAACAAGAATTTTCAGT	Copper Metallochaperone	Odermatt and Solioz (1995)
CopZ2	TCAAATCACCTCTGCTTGAT		

Chromosomal DNA from *Methylosinus trichosporium* OB3b was digested to completion with *Eco*RI, *Sal*I or *Sst*I, run through a 0.8 % TBE agarose gel and transferred to Nylon membrane by Southern transfer. These blots were then challenged with ^{32}P-labelled *cop* gene specific probes. Even at the lowest stringency tested (2 x SSC, room temperature washes) no DNA fragments were highlighted with any of these probes. This suggested that any copper transport genes present in the genome of *Methylosinus trichosporium* OB3b were substantially different from that of the *cop* operon used as probes in these experiments.

Enterococcus hirae is a Gram-positive enteric pathogen and so in hindsight it may have been a poor candidate for heterologous probing experiments, even though it was probably the best characterised set of bacterial copper homeostasis genes. Any future attempts should be made using copper transport operons from more closely related organisms, such as the newly characterised *Escherichia coli cue, cop,* and *cus* operons; these were not available at the time of these experiments (Munson *et al.,* 2000; Outten *et al.,* 2000; Outten *et al.,* 2001; Petersen *et al.,* 2000;Grass and Rensing, 2001). However, the best chance for identification of copper transport operons from methanotrophs is presented by the imminent arrival of the *Methylococcus capsulatus* Bath genome sequence. Indeed a search of the unfinished genome revealed several possible cation transport proteins (Professor Harald Jensen, University of Bergen, Norway, personal communication), suggesting that the study of copper transport in methanotrophs may be best served by using *Methylococcus capsulatus* Bath as a model system.

7.3.2 Heterologous probing of the chromosome of *Methylosinus trichosporium* OB3b with *mopE* from *Methylococcus capsulatus* Bath

Recent research on outer membrane proteins from *M. capsulatus* Bath revealed the presence of a protein, MopE, which is expressed in the outer membrane of *M. capsulatus* Bath and cleaved at a specific site before secretion into the growth medium at high levels. This occurs both in the absence of copper and to a lesser extent at higher copper levels (Professor Harald Jensen, University of Bergen, Norway, personal communication). It has also been shown that MopE is a copper-binding protein (Ladstein, 1999). Analysis of the *mopE* gene revealed its N-terminal region had homology with the *corA* gene from *Methylomicrobium album* BG8, a copper repressible protein of unknown function (Berson and Lidstrom, 1997; Fjellibirkeland *et al.,* 2001). Thus, it is possible that MopE is in some way involved in copper transport. The aim of this section was to determine if the gene encoding this protein, *mopE,* was present in the genome of *Methylosinus trichosporium* OB3b and other methanotrophs and to test if antibody raised against the *Methylococcus capsulatus* Bath MopE cross-reacted with any proteins in membrane and spent media fractions from cultures of *Methylosinus trichosporium* OB3b.

Chromosomal DNA was extracted from 6 methanotrophs: *Methylocystis* sp. strain M, *Methylocystis parvus* OBBP, *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3b, *Methylomonas* sp. S1 and *Methylomonas rubrum*. Approximately 5 μg DNA were digested with *Bam*HI, *Eco*RI or *Sal*I before separation on a 0.8% TBE-agarose gel and Southern transfer to nylon membrane. This blot was then probed with a *mopE*-specific PCR derived probe. The entire *mopE* gene was first amplified from the chromosome of *Methylococcus capsulatus* Bath using the primers mopEF (ACCATGAACGAAAAGCATTGC) and mopER (CGGCTTGGAGATCGTGATG) before being cloned in to the vector pCR2.1-TOPO and sequenced to verify its identity. The probe was then generated by re-amplification of *mopE* from this plasmid. It is clear from these blots that the *mopE* gene from *Methylococcus capsulatus* Bath highlighted bands in all methanotrophs tested at medium stringency (1xSSC, 60°C; Figure 7.4). However, all bands except for those from *Methylocystis* sp. strain M were lost on washing at relatively high stringency (80°C, 1xSSC, data not shown). These data suggest that genes similar to *mopE* may be present in all methanotrophs tested. Unfortunately, the DNA stock from *Methylomicrobium album* BG8 used in this experiment actually originated from *Methylosinus trichosporium* OB3b, but this organism should be tested in the future since *mopE* has similarity to the *corA* gene (Fjellibirkeland *et al.*, 2001).

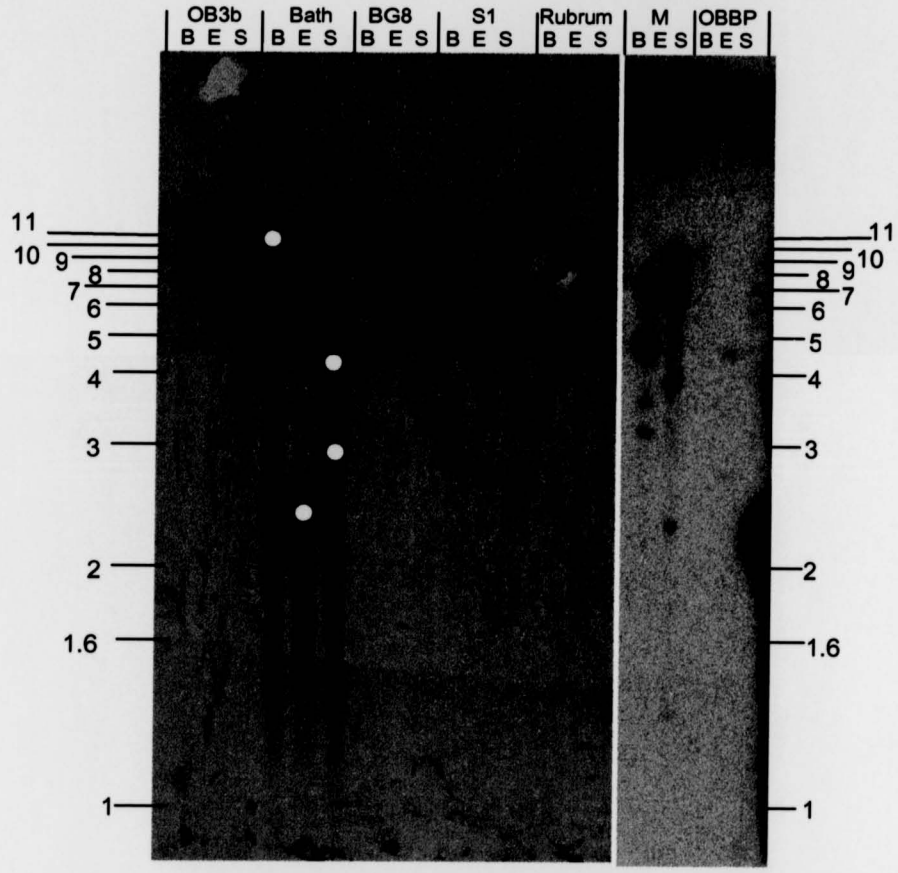


Figure 7.4 Probing of the chromosome of 6 methanotrophs with the *mopE* gene from *Methylococus capsulatus* Bath.

A *mopE* probe was generated by PCR using the primers mopEF (ACCATGAACGAAAAGCATTGC) and mopER (CGGCTTGGAGATCGTGATG) and used to probe chromosomal DNA (5µg) from: OB3b, *Methylosinus trichosporium* OB3b; Bath, *Methylococus capsulatus* Bath; BG8, *Methyomicrobium album* BG8; S1, *methymonas* S1; Rubrum, *Methylomonas rubrum*; M, *methylocystis* sp. strain M; OBBP, *Methylocystis parvus* OBBP. The DNA was digested to completion with *Bam*HI (B), *Eco*RI (E) and *Sal*I (S) before transfer to nylon membrane before hybridisation. This blot was washed with 1 xSSC at 60°C before exposure to X-ray film for 48h hours at –80C. The bands highlighted with yellow circles were visible after just 4 hours exposure, but appear over-exposed in this diagram.

The *mopE* gene product, MopE, is a 60 kDa outer membrane protein which is cleaved at a specific site to release a 45 kDa truncated version named ΔMopE into the medium (Fjellibirkeland *et al.,* 2001). It can clearly be detected in the cell supernatant and outer membrane fractions of chemostat grown cultures of *Methylococcus capsulatus* Bath by Western blotting using MopE specific antibodies. Therefore, cell supernatants and outer membrane fractions were prepared from *M. trichosporium* OB3b in order to determine the presence of MopE. These fractions were separated using SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. After challenging these blots with (*Methylococcus capsulatus* Bath), MopE-specific antibodies (a gift from Professor Harald Jensen, University of Bergen) no proteins were found to cross-react with the cell supernatant or membrane fractions from *Methylosinus trichosporium* OB3b in two separate experiments (data not shown). These results suggest that while *Methylosinus trichosporium* OB3b may possess a *mopE*-like gene, identifiable in Southern blotting, it either does not express a MopE protein or that the MopE from *M. trichosporium* OB3b does not cross-react with antibody raised against MopE from *M. capsulatus* Bath. It would be interesting to use these antibodies against cell fractions of *Methylomicrobium album* BG8, although these may also not cross-react with the antibodies raised against MopE from *Methylococcus capsulatus* Bath.

7.4 A study of protein expression of *Methylosinus trichosporium* OB3b in response to copper: a proteomic approach

The proteome is a term used to describe the full complement of proteins expressed at a given time and is visualised using established 2D-Gel electrophoresis techniques. Thus, it can be used to assess which proteins are 'up-or down-regulated' in response to a specific stimulus. In these experiments the proteome of *Methylosinus trichosporium* OB3b was assessed using chemostat grown cells from high and low copper media. The following 2D-PAGE experiments were carried out in the laboratory of Professor Harald Jensen, University of Bergen, Norway, in collaboration with Frøde Berven and Odd-André Karlsen.

Chemostat cultures of *Methylosinus trichosporium* OB3b were grown in a 4l vessel with a flow rate of 0.03 h^{-1}, and attained a typical $OD_{540} \sim 8$. Low-copper media contained no added copper, and high copper media 0.1 mgl^{-1} (added as $CuSO_4$). Samples were removed (200 ml) and concentrated by centrifugation before resuspension in 10 ml of 20 mM Tris-HCl (pH 7.3), and drop-frozen in liquid-nitrogen. Samples (1g wet-weight) were then fractionated according to the method described in section 2.21. SDS-Page of these samples clearly shows that the soluble fractions from the low-copper grown cells contained the three subunits of the sMMO hydroxylase component, but were absent from the corresponding high copper and membrane fractions (Figure 7.5). However, these gels only allow differentiation of expression levels between the two copper concentrations in cases where a particular protein is expressed at very high levels or not at all, i.e in the case of sMMO hydroxylase.

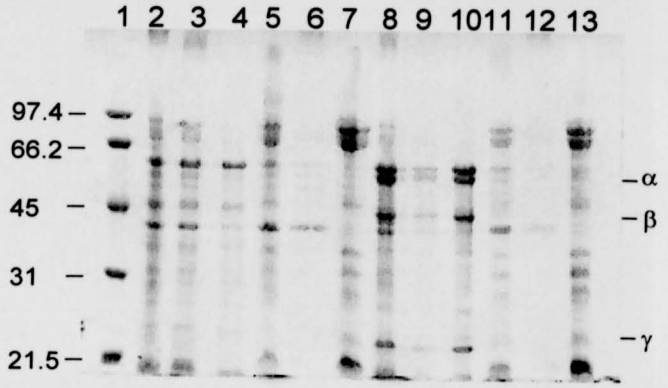
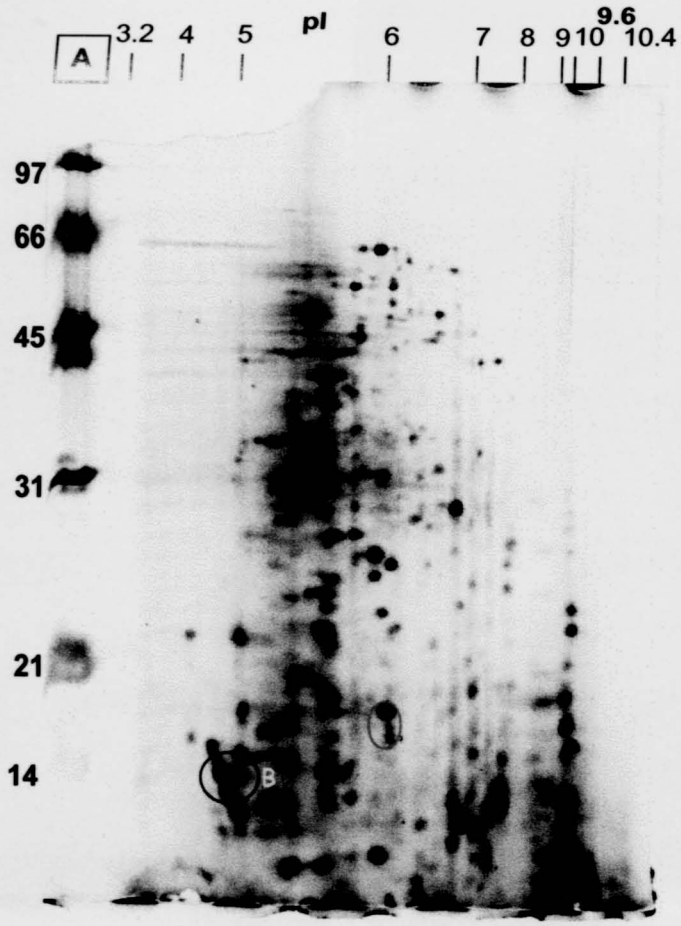


Figure 7.5 Coomassie stained SDS-PAGE of cell fractions from *Methylosinus trichosporium* OB3b. Lanes 2-7 are from cells grown under high copper conditions, and 7-13 low copper. Lanes 1: Low molecular mass marker; 2 & 8: 5 ml whole lysate; 3: 5 ml cell-free extract; 3 & 10: 5 ml Cytoplasm; 4 & 11: 10 µl total membrane; 5 & 12: 10 ml inner membrane; 6 & 13: 10 ml outer membrane; 9 empty. Molecular markers are shown in kDa to the left of the gel; the three sMMO hydroxylase subunits are indicated on the right.

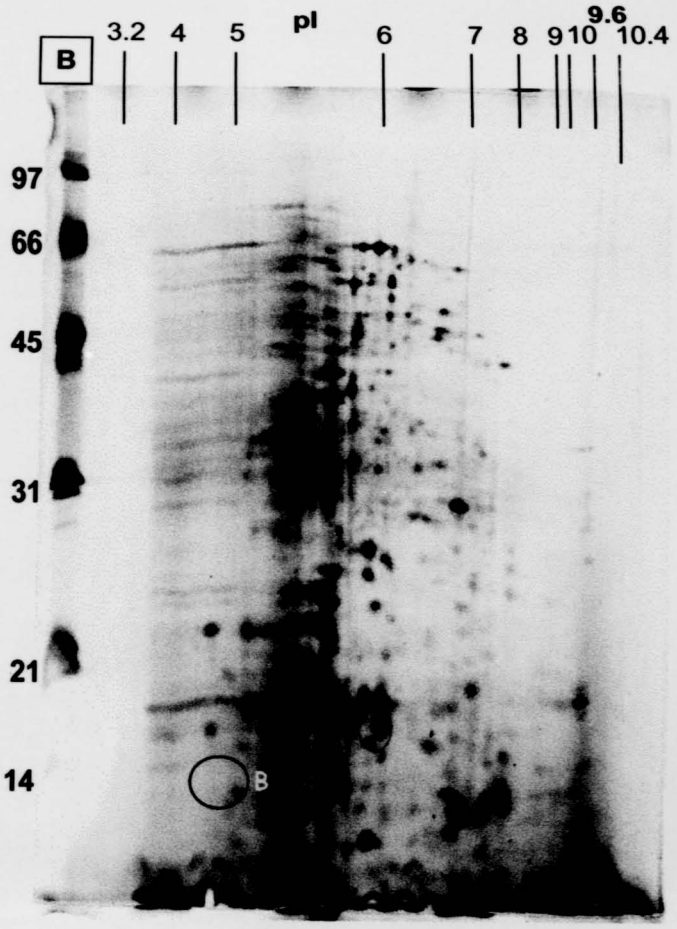
In order to identify further differences between the proteins expressed in cells grown under these two conditions, approximately 100 µg protein from the cytoplasmic, total membrane, outer membrane and inner membrane fractions of *Methylosinus trichosporium* OB3b were separated by 2D-gel electrophoresis and visualized by silver-staining, as described in section 2.22.

Unfortunately, problems were encountered in the resolution of proteins from the cytoplasmic fractions (Figure 7.6A, 7.6B). The acidic region of these gels is heavily streaked and the basic portion is also poorly resolved. The most likely reason for this problem was a failure to remove enough salt during the sample preparation process. Future resolution of the cytoplasmic fraction may be achieved by introducing additional cell washing steps before fractionation is performed. However, these gels do allow the resolution of some polypeptides, and clear differences are obvious for three spots. For example, the pair of proteins labelled B (Figure 7.6A, 7.6B)(pI 4.5, MW = 14kDa) may represent protein B (MmoB), and are absent from high copper extracts. Also of note is the apparent absence of the sMMO hydroxylase polypeptides, which were highly visible in the low copper lanes from the SDS-PAGE gels of cytoplasmic fractions (Figure 7.5). A Western blot of the cytoplasmic fractions was probed with antibody specific for the sMMO hydroxylase subunits but

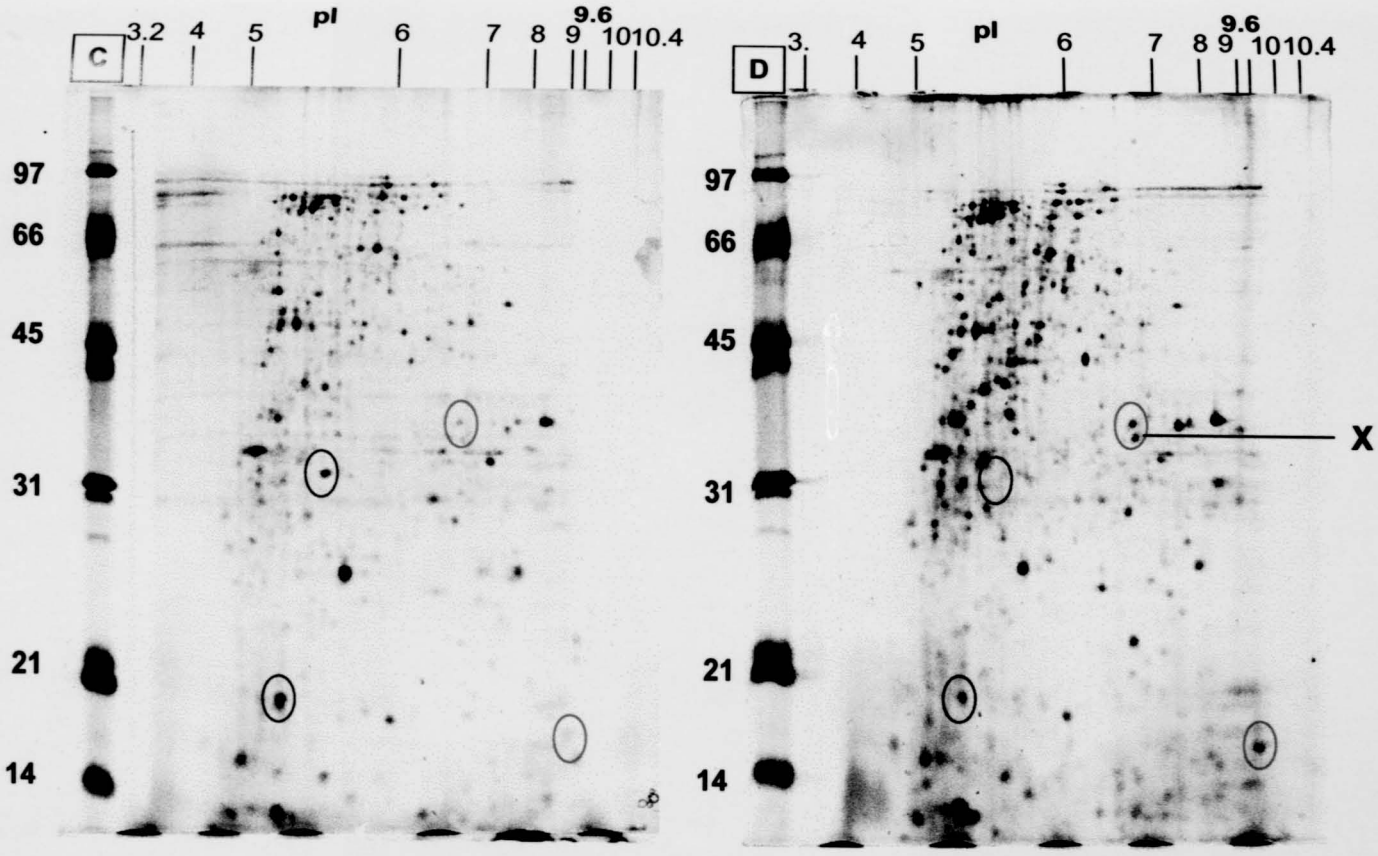
this failed to highlight any specific polypeptide spots (data not shown). However, repetition of these gels using an amended protein preparation method may allow an improvement in the resolution of the cytoplasmic fraction.



Low copper: cytoplasmic

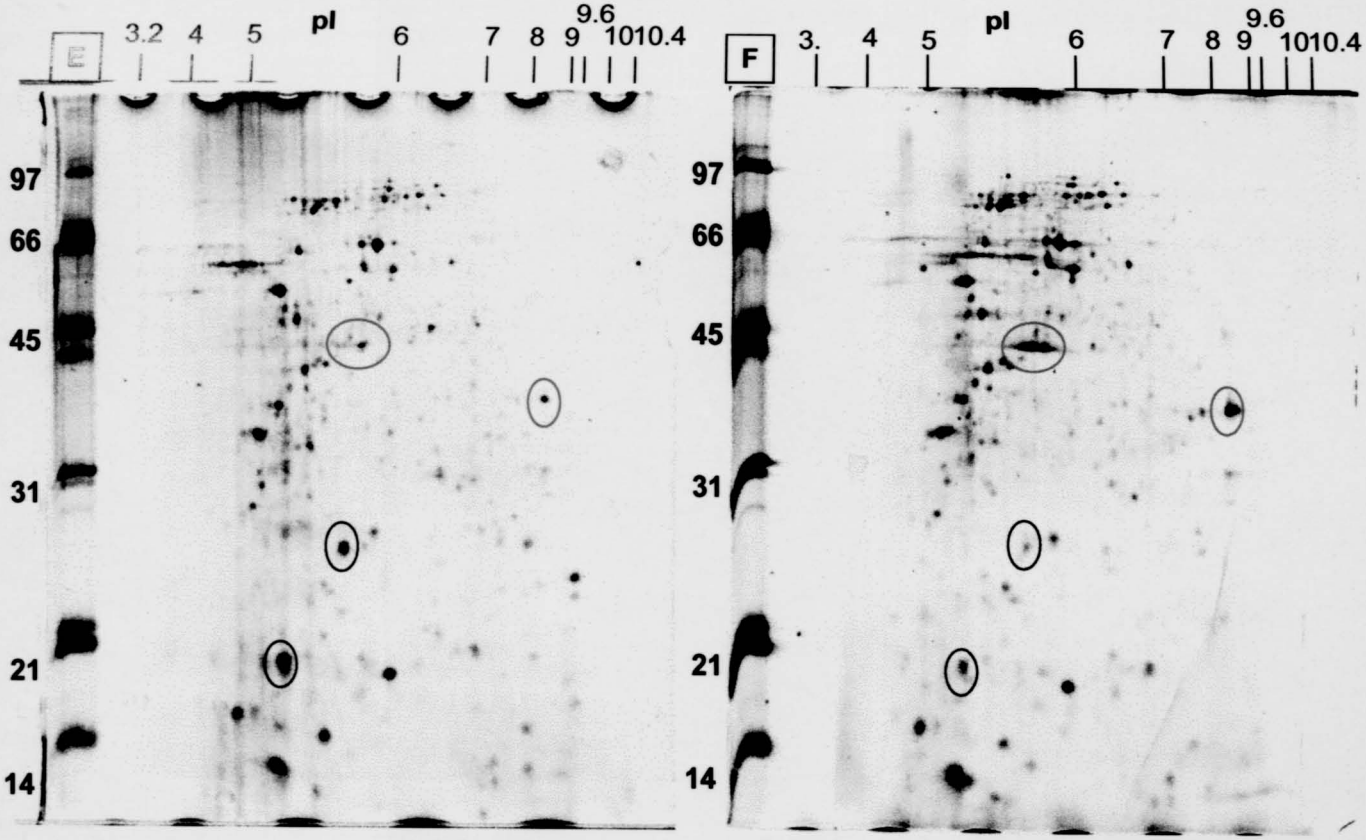


High copper: cytoplasmic



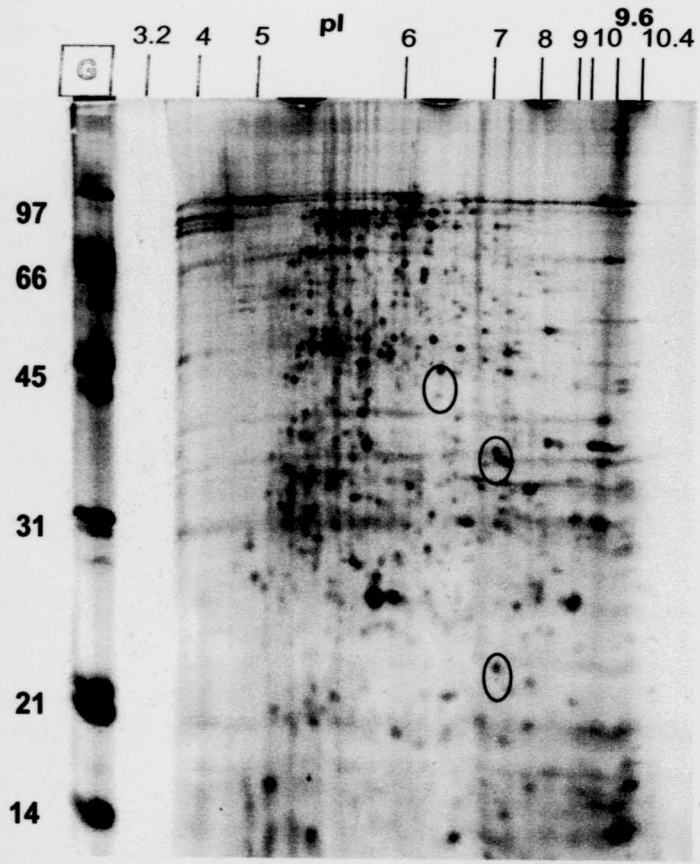
Low copper: Total membrane

High copper: Total membrane

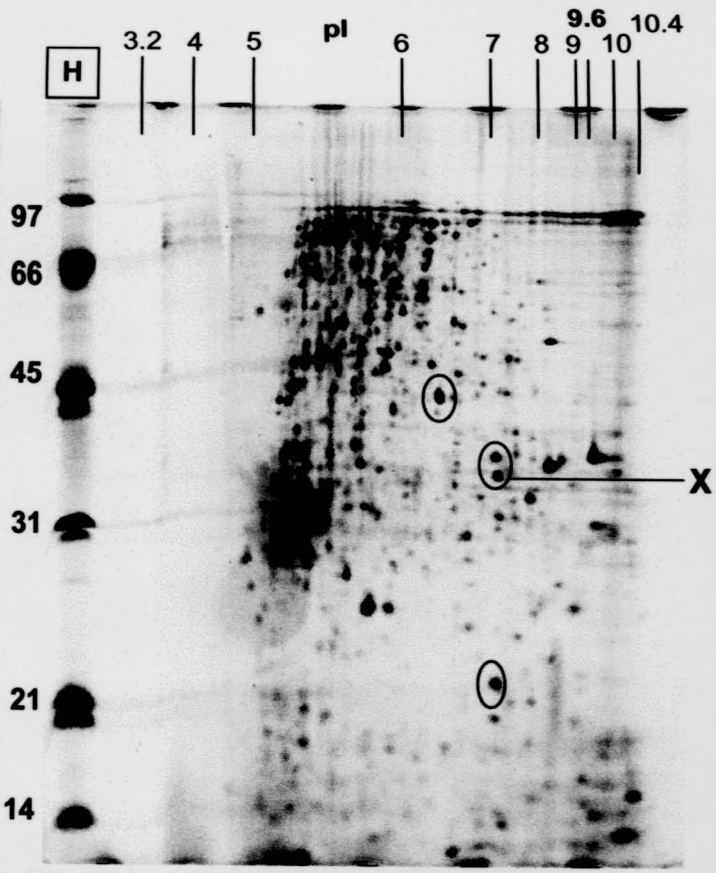


Low copper: Outer membrane

High copper: Outer membrane



Low copper: Inner membrane



High copper: Inner membrane

Figure 7.7 2D gels of fractionated cell extracts from *Methylosinus trichosporium*OB3b.

Cell extracts were prepared as described in section 2. 22.1 from cells grown in 4 l chemostats (D=0.3h-1, OD540~8). Low copper media contained no added copper and high copper media contained 0.1 mgl^{-1} (added in as $CuSO_4$). The first dimension, isoelectric focusing, was performed using 13 cm pH 3-10 Non-linear IPG strips from Amersham Pharmacia. Proteins were then separated by molecular mass using a 12 % SDS gel. Silver staining was performed as described in section 2.2.3. Protein molecular masses are shown in kDa. pI units were calculated using manufacturers specifications. Polypeptide spots circled in red appear more highly expressed on low copper medium and those highlighted in blue are more highly expressed in high copper medium.

Table 7.3 Predicted molecular masses and isoelectric points (pI) for the known components of the sMMO and pMMO enzymes. Predicted molecular weight and pI were calculated using the Clone manager 5 package from SECentra. Only the predicted values for the fully sequenced copy of the pMMO operon (Gilbert *et al.*, 2000) are shown.

Protein	Gene	Predicted M_w	Predicted pI
α-subunit of sMMO hydroxylase	*mmoX*	59954	5.95
β-subunit of sMMO hydroxylase	*mmoY*	45020	8.33
γ-subunit of sMMO hydroxylase	*mmoZ*	19326	9.16
sMMO regulatory protein	*mmoB*	14883	4.37
sMMO reductase	*mmoC*	36732	4.83
PmoC	$pmoC_1$	29003	5.17
PmoA	$pmoA_1$	28503	8.19
PmoB	$pmoB_1$	46883	5.92

In contrast to the cytoplasmic fractions, the membrane fractions prepared by the method described in section 2.22.1 were very successful. Both the total and outer membrane fractions on these gels were well resolved and allowed the separation of over a hundred polypeptide spots (Figures 7.6C-H). However, it was not possible to identify the pMMO subunits, based on predictions of mass and isoelectric point (pI) from sequence data (Table 7.3). A comparison of both the total and inner membrane fraction from the high and low copper grown cells is presented in figures 7.6C-H. Several polypeptide spots have strikingly different expression patterns in response to copper concentrations. In particular, a pair of polypeptides (labelled X: pI 6.9; M_W = 40 kDa), seems to be markedly up-regulated in the high copper samples when compared to low copper but is only present in the enriched total and inner membrane fractions but not the outer membrane fraction, suggesting they reside in the inner membrane of *Methylosinus trichosporium* OB3b.

In future studies, these differentially expressed proteins may prove interesting targets for sequencing by mass spectrometry methods and may reveal new components involved in the response to copper in this organism. What is clear from these gels is that it is possible to separate a large number of polypeptides from *M. trichosporium* OB3b by 2D-PAGE, revealing differences in the suite of expressed polypeptides in cells grown on high and low copper. It also allows the separation of outer and inner membrane fractions, as exemplified by protein pair X This

preliminary study has allowed the identification of several polypeptides whose expression differs between high and low copper grown cells and should form a useful base for future studies of the copper switch in this organism.

7.5 Discovery of a copper reductase activity in *Methylosinus trichosporium* OB3b

Methanotrophs are a free-living obligately aerobic group of organisms which are found in many environments. It is known, that like many other organisms, they have a requirement for copper. In addition to that required for enzymes such as superoxide dismutase and terminal respiratory oxidases, they also require copper for the functioning of the particulate methane monooxygenase enzyme (pMMO). Indeed, studies have shown that the pMMO enzyme co-purifies with 14.5 copper atoms per active complex (Nguyen *et al.*,1994; Zahn and Dispirito, 1996). It is also thought that the predominant ionic form of copper, found in association with the pMMO enzyme, is the reduced cuprous (Cu [I]) form (Nguyen *et al.*, 1994; Takeguchi *et al.*, 1998). However, methanotrophs live in aerobic environments where the predominant form of copper is the cupric (Cu [II]) state. Indeed, under such conditions the cuprous form is a short-lived species, being spontaneously oxidised to the cupric form as soon as it appears (Greenwood and Earnshaw, 1984). Thus, these organisms must be able to reduce cupric copper (Cu[II]) to cuprous copper (Cu[I]) during the uptake process.

In order to try and gain an insight in to copper transport mechanisms in this organism, a copper reductase assay was developed from a method employed by Georgatsou *et al.*, (1997). The assay relies on the reduction of copper from its cupric to cuprous form and the stabilisation of the copper [I] species by the presence of a specific copper [I] chelator. This assay is described in section 2.24 and used the copper [I] chelator bathocuproine disulfonic acid (BCS). The formation of this complex can be followed in a spectrophotometer at 482 nm. Approximate rates of copper [I] formation are given as $nmolsmin^{-1}$ per 10^9 cells, and calculated using an extinction coefficient of 12.25 $mM^{-1}cm^{-1}$ at 482nm (see section 2.24). Cells were grown in NMS (containing 4 μM copper) in all experiments presented here unless otherwise stated. Negative controls containing Reductase assay solution, copper sulphate and BCS in the absence of cells were performed in all cases and these values

were subtracted from the values gained for the cell assay in order to permit the calculation of approximate reaction rates.

Figure 7.7 shows the increase in absorbance observed with the formation of the Cu[I]-BCS complex grown in NMS (4 μM copper [II]) over time. The approximate reaction rates from these data are shown in Table 7.4, and the method of calculation described in section 2.24. These data show that this organism does indeed possess a copper reducing activity. However, this activity could originate from a cell-associated chemical reaction. Therefore, an additional control was included in which a cell sample was boiled for 15 min before assaying for copper reducing activity. This control gave the same rate of copper reduction as that observed for the spontaneous chemical reduction controls and confirmed that the observed activity was a biological cell-associated enzyme activity.

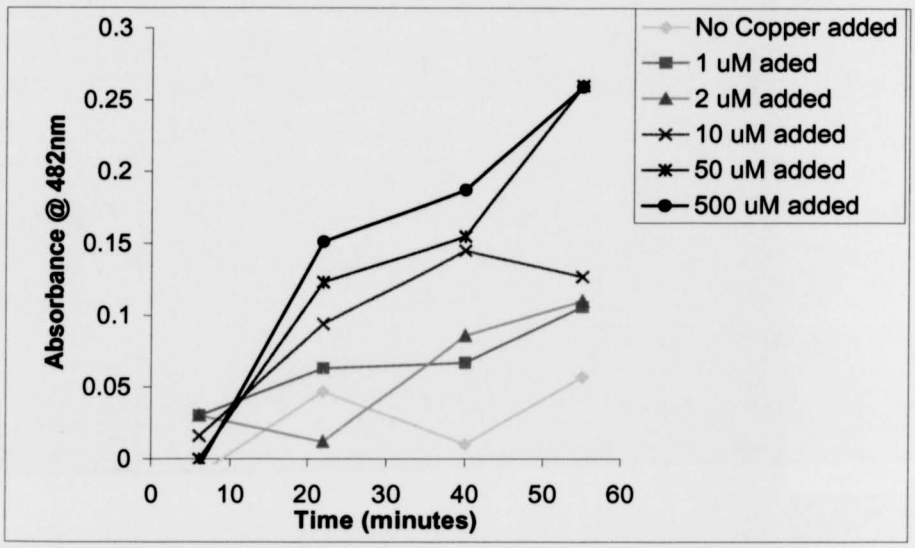


Figure 7.8 Copper reductase activity of *Methylosinus trichosporium* OB3b cells.
The formation of a copper[I]-Bathocuproinedisulfonicacid complex catalysed by whole cells followed spectrophotometrically at 482nm as described in section 2.24. This figure represents data from one set of assays.

Table 7.4 Copper [II] reduction rates of *Methylosinus trichosporium* OB3b. The cells used in these assays were grown in the presence of 4mM $CuSO_4$. Rates were calculated as described in section 2.? Using an Extinction coefficient $12.25mM^{-1}cm^{-1}$ at 482 nm. The slope of the lines shown in figure 7.7 was estimated using the linear regression tool from the Microsoft Excel package.

Copper concentration in assay mixture (μM)	Copper [II] reduction rate ($nmolsmin^{-1}per10^9$ cells)
0	0.01
1	0.11
2	0.16
10	0.20
50	0.40
500	0.41

The maximum rate of copper [II] reduction achieved in these experiments was 0.4 $nmolmin^{-1}/10^9$ cells (Table 7.4), and this rate was observed when either 50 μM or 500 μM (final concentration) was included in the assay mixture. The rates observed for the lower substrate concentrations are more difficult to estimate from these data and therefore a K_m for this reaction is difficult to estimate, but is between 1 μM and 50 μM. A repetition of this assay gave reduction rates of approximately half the level of the data shown in Figure 7.7 and table 7.4 but still had a maximum reduction rate at 50 μM and 500 μM (data not shown).

In this section, data were presented showing the development of a copper reductase assay for *Methylosinus trichosporium* OB3b and its successful use in the detection of a copper reductase activity. This suggested a mechanism for copper uptake in this organism, which involves the reduction of copper [II] to copper [I] during uptake. However, this experiment has not been tested in cells grown under copper starvation conditions and consequently there is no knowledge of how it may be regulated. The variation in the rates of reduction achieved in separate experiments may prove a problem in future experiments and hinder a detailed characterisation of this enzyme activity. These variations may be due to subtle differences in the state of the batch cultures and may be overcome by the use of chemostat-grown cells.

7.6 Discussion

The aim of the experiments presented in this chapter was to identify gene products which are involved in the regulation of the 'copper-switch', in addition to the known members of the *pmo*, *mmo* and *rpoN* gene clusters.

The first and potentially most powerful method employed was transposon mutagenesis. However, several problems were encountered which unfortunately made this method difficult. The conjugation frequencies recorded in this study are low but comparable to those observed previously for *Methylosinus trichosporium* OB3b (Martin and Murrell, 1995; Lloyd *et al.,* 1999a), being in the range from $1x10^{-7}$ to 1×10^{-9}. However, this is not the major reason for the failiure of this method as even at these frequencies a transposon library of 5,000 – 10,000 could realistically be constructed. The frequency of spontaneous resistance to streptomycin, however, is also in the range of $1x10^{-7}$ to 1×10^{-9}. More importantly the non-random insertion of transposons into the genome of *Methylosinus trichosporium* OB3b means that a library of these transposon insertions would be useless for the identification of genes involved in the copper switch (or any other system).

The importance of copper to *Methylosinus trichosporium* OB3b is well known, and evidence is emerging that dedicated copper transport systems exist in methanotrophs. Dispirito *et al.,* (1998), purified small highly modified octapeptide copper-binding compounds from the growth medium of both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath. Several sMMO-constitutive mutants created by chemical mutagenesis of *Methylosinus trichosporium* OB3b appear to be defective in the utilisation of these copper binding compounds (Fitch *et al.,* 1993; Dispirito *et al.,* 1998) suggesting that a dedicated system exists for the utilisation of these compounds in these organisms. Thus, an attempt was made to identify components of the copper transport system for *Methylosinus trichosporium* OB3b. Heterologous probing using genes from the *cop* operon of *Enterococcus hirae* failed to identify homologues within the genome of *Methylosinus trichosporium* OB3b. However, such copper transport systems are almost definitely present in this organism. Indeed, a preliminary screen of the genome sequence of the methanotroph *Methylococcus capsulatus* Bath at the TIGR Blast server of unfinished genomes reveals the presence of many metal ion transport proteins.

Further heterologous probing experiments presented here show the possible presence of a gene with similarity to the *mopE* gene from *Methylococcus capsulatus* Bath in several methanotrophs. This encodes MopE, which resides in the outer membrane of *Methylococcus capsulatus* Bath as a 64 kDa polypeptide and is cleaved to give a 46 kDa polypeptide that is released into the culture medium in large amounts (Fjellibirkeland *et al.,* 2001). This protein has been implicated to be involved in copper transport since its C-terminal has significant homology to the derived amino-acid sequence of the copper-repressible gene, *corA*, from *Methylomicrobium album* BG8 (Berson and Lidstrom, 1997). MopE has also been shown to bind copper (Ladstein, Msc thesis) and is expressed at higher levels under copper limitation (Harald Jensen, personal communication). These data suggest that this gene product may be involved in copper metabolism in methanotrophs. The mutation of the *mopE* gene from *Methylococcus capsulatus* Bath is imperative if its function is to be established.

In this Chapter, preliminary evidence for the presence of a copper reductase system is presented for *Methylosinus trichosporium* OB3b. This suggests a reductive mechanism for copper uptake in this organism. Such copper reductase systems have been found in only two bacteria, *Enterococcus hirae* (Wunderli-Ye and Solioz, 1999) and *E. coli* (Rapisarda *et al.,* 1999) and in eukaryotic organisms such as *Chlamydomonas reinhardtii* (Hill *et al.,* 1996), *Cryptococcus neoformans* (Nyhus & Jacobson, 1999) and *Saccharomyces cerevisiae* (Hassett & Kosman, 1995; Georgatsou *et al.,* 1997). Indeed, an *E.coli* strain deficient in the copper reducing NADH-linked copper reductase (NDH-2) grows more slowly than the wild-type strain in copper-depleted medium, indicating that copper reductase enzymes may be an important component of the copper transport system in *E. coli* (Rapisarda *et al.,* 1999). In *Saccharomyces cerevisiae* there appear to be two copper reducing enzymes encoded by Fre1p and Fre2p, which also reduce ferric iron (Fe[III]) to ferrous iron (Fe[II]) and whose expression is induced by both iron and copper depletion (Hassett & Kosman, 1995; Georgatsou *et al.,* 1997). In addition, it is thought that the intracellular form of copper found in *Methylosinus trichosporium* OB3b is predominantly the cuprous (Cu [I]) form (Nguyen *et al.,* 1994; Takeguchi *et al.,* 1999a,b) suggesting that copper reduction may be an important step in the transport of copper into the cells. Future studies should focus on the improvement in reproducibility of the assay and the study of this activity in chemostat-grown cultures

of *Methylosinus trichosporium* OB3b grown under 'low' and 'high' copper conditions.

Finally, the preliminary proteomic study of *Methylosinus trichosporium* OB3b shown in this Chapter coupled with similar experiments performed on the proteome of *Methylococcus capsulatus* Bath by our collaborators in the Jensen group (University of Bergen, Norway), show that there are significant differences in the suite of proteins expressed in response to copper in methanotrophs. The recent advent of mass spectrometry techniques, enabling sequence information to be gained from the protein spots identified on silver stained 2D-gels, should allow the identification of members of the suite of proteins expressed in response to copper. In fact, at the present time, the proteomic approach, coupled with the information gained from the *Methylococcus capsulatus* Bath genome sequence, represents the best chance to identify components of a copper responsive regulon.

CHAPTER 8

FINAL DISCUSSION

Chapter 8: Final Discussion

A detailed discussion of the results presented has been conducted within the relevant chapter and so I present here a summary of the major findings in this thesis and a brief final discussion of the implications of the work as a whole.

8.1 Summary of major findings

Chapter 3:

- Primer extension allowed the identification of the transcriptional start for one of the *pmoCAB* operons from *Methylocystis* sp. strain M and suggests it is transcribed as a polycistronic mRNA molecule containing the *pmoCAB* genes.
- Tandem σ^{70} promoters lie 5' of the *pmoCAB* operon in *Methylocystis* sp. strain M and $pmoCAB_2$ (but not $pmoCAB_1$) of *Methylosinus trichosporium* OB3b.
- RT-PCR has allowed the localisation of the transcriptional start site for the *pmoCAB* operons of *Methylosinus trichosporium* OB3b to a region of sequence homology between promoters 5' of *pmoCAB* from *Methylosinus trichosporium* OB3b and *Methylocytis* sp. strain M.

Chapter 4:

- Transcription of *pmoCAB* from *Methylosinus trichosporium* OB3b was detected by RT-PCR from cells grown in the presence of high and low concentrations of copper using methane or methanol as a sole carbon and energy source. This suggests that a basal level of *pmoCAB* transcription occurs under all growth conditions.
- RT-PCR results suggest that transcription of the *mmoXYBZorfYmmoC* operon may be regulated in response to carbon source (i.e. methane/ methanol) as well as copper.

Chapter 5:

- The *rpoN* gene cluster was cloned and sequenced from *Methylosinus trichosporium* OB3b.
- *rpoN* lies within a typical *rpoN* cluster and phylogenetic analysis reveals that it is most closely related to the *rpoN* from members of the family *Rhizobiaceae.*
- An *rpoN* marker-exchange mutant of *Methylosinus trichosporium* OB3b was constructed to analyse its function.
- The *rpoN* gene product, σ^N, is required for the expression of at least three important systems: 1. The soluble methane monooxygenase.
  2. Nitrate utilisation.
  3. Nitrogen fixation.
- The *rpoN* mutant lacked *mmoX* specific transcripts indicating that σ^N acts at the level of transcription (Figure 8.1).
- Expression of the genes encoding pMMO and GS/GOGAT are σ^N-independent.

Chapter 6:

- Sequence analysis of the region 5' of the *mmo* operon (sequenced by Ian McDonald) revealed the presence of genes encoding a GroEL chaperone ($groEL_{mmo}$) and an EBP transcriptional activator (*mmoR*).
- An *mmoR* marker-exchange mutant of *Methylosinus trichosporium* OB3b was constructed to analyse its function (Julie Scanlan).
- Phenotypic analysis of the *mmoR* mutant revealed that it lacked active sMMO enzyme and acted on *mmoX* expression at the level of transcription (Figure 8.1).
- Western analysis using sMMO-hydroxylase specific antibody on cell-free extracts from the *mmoR* and *rpoN* mutants indicated that a promoter located between *mmoX* and *mmoY* may be active under the conditions tested.

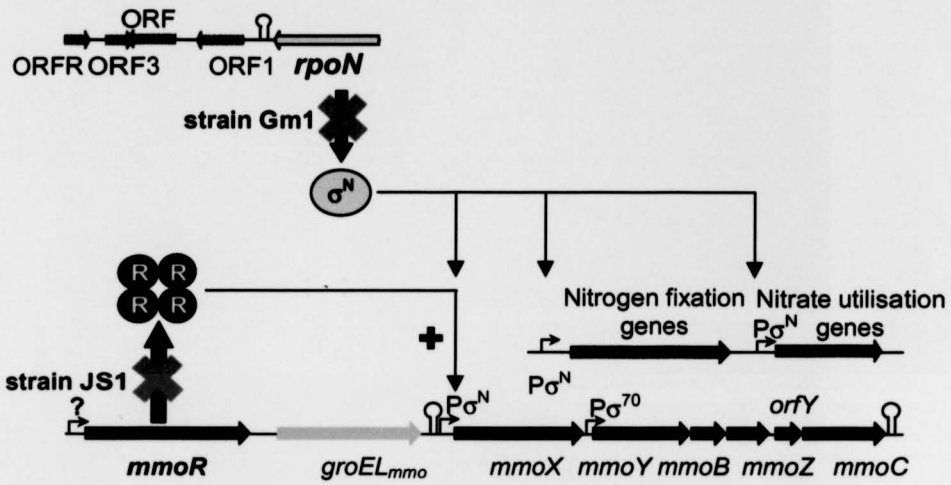


Figure 8.1 Sumary of effects of mutations in *rpoN* and *mmoR* genes from *Methylosinus trichosporium* OB3b (strains Gm1 and JS1) in the absence of copper.
Genes inactivated in strains Gm1 and JS1 are indicated by red crosses. The *mmo* cluster promoters identified by primer extension are shown (Nielsen *et al.*, 1997). Nitrogen fixation and nitrate utilisation genes are shown with σ^N promoters due to the phenotypes of strain Gm1, summarised in Chapter 6. The *mmoR* gene product is represented as a tetramer (R).

Chapter 7:

- A search for new members of a 'copper-regulon' was conducted using Tn5 mutgenesis and heterologous probing for copper transport genes, but proved unsuccessful.
- Heterologous probing for the gene encoding MopE, which has been implicated in copper transport, revealed hybridising bands in 6 methanotrophs.
- A copper reductase activity has been identified in *Methylosinus trichosporium* OB3b, suggesting a reductive copper uptake system.
- A preliminary proteomic study revealed several target proteins, which are differentially expressed in response to changing copper levels.

8.2 An improved model for the regulation of the *pmo* and *mmo* gene operons in *Methylosinus trichosporium* OB3b

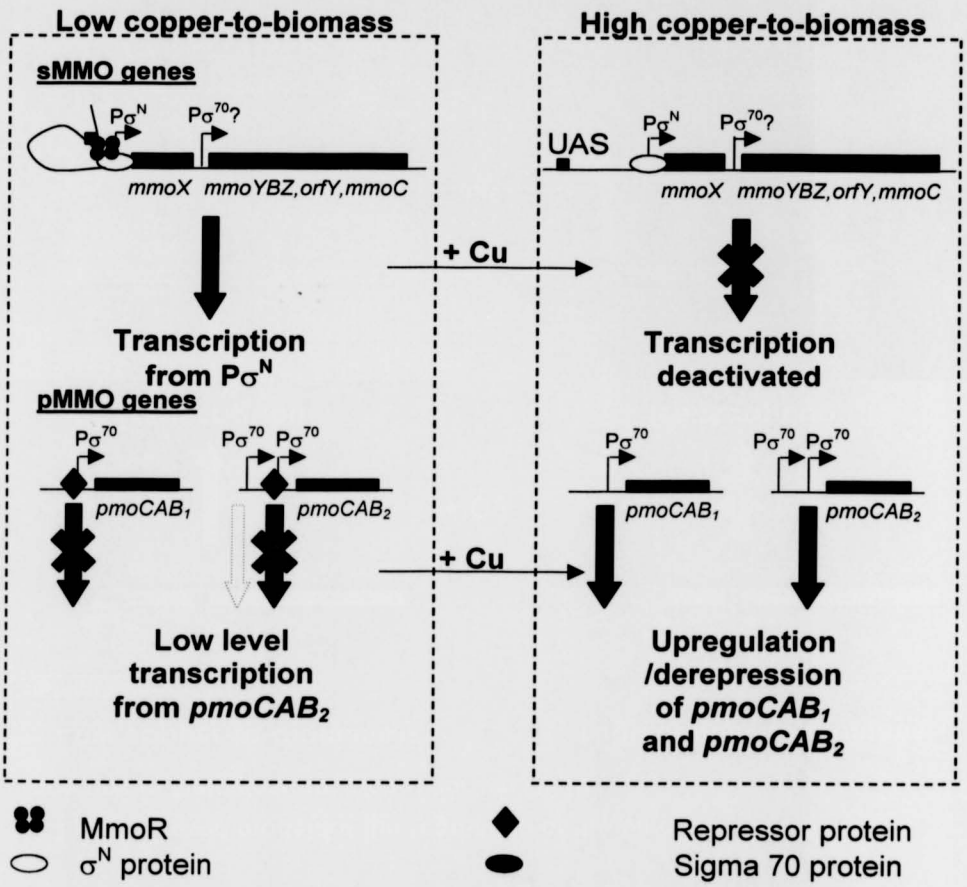


Figure 8.2. Hypothetical model for the regulation of methane monooxygenase genes by copper in *Methylosinus trichosporium* OB3b.
In cells growing under low-copper to biomass ratios, MmoR binds to an upstream activating sequence (UAS) and directly interacts with the σ^N protein, bound at $P\sigma^N$, in a mechanism proposed to involve looping of the DNA between the UAS and $P\sigma^N$. This directs the formation of an open complex and transcription of the sMMO genes then occurs. At the same time, an unknown repressor incompletely represses transcription of the pMMO genes, allowing a basal level of *pmo* transcription under low copper conditions. On the addition of copper, sMMO gene transcription becomes deactivated and pMMO gene transcription is derepressed, resulting in upregulation of *pmo* transcription from $P\sigma^{70}$.

The overall aim of the work conducted in this thesis was to increase the understanding of the unique 'copper switch' which lies at the heart of the regulation of the methane monooxygenase genes in several methanotrophic bacteria. It was first discovered by Stanley *et al.,* (1983), who established that copper controlled the

expression of the soluble and particulate methane monooxygenase enzymes. However, not until the work of Nielsen *et al.,* (1997) had a model for this unique system been proposed. The results summarised in section 8.1 have increased our understanding of the regulation of the copper switch at the molecular level in *M. trichosporium* OB3b and allow the construction of an improved model based on that of Nielsen *et al.,* (1997). This is presented in Figure 8.2.

Under low-copper to biomass ratios, the sMMO enzyme is transcribed from the $P\sigma^N$ promoter. Several pieces of experimental evidence support this part of the model. Firstly, a transcriptional start site 5' of *mmoX* which corresponds to $P\sigma^N$ has been mapped by primer extension (Nielsen *et al.,*1997). Secondly, inactivation of both the *mmoR* and *rpoN* genes by marker-exchange mutagenesis resulted in the creation of two strains of *M. trichosporium* OB3b, JS1 and Gm1 respectively, which are incapable of transcription of the *mmoX* gene, lack the α-subunit of the sMMO-hydroxylase and possess an inactive sMMO enzyme. Thus, it is clear that the *mmo* operon is transcribed in response to low copper levels in a σ^N and MmoR-dependent manner. MmoR is a member of the Enhancer Binding Protein (EBP) family of transcriptional activators, so called due to the fact that they bind to upstream activator sequences (UAS) which act as enhancers of transcription from sites remote to their cognate promoter (Morrett and Segovia, 1993; Buck *et al.,* 2000). However, the UAS sequence for MmoR has yet to be defined, although DNA-footprinting experiments using purified MmoR should allow the identification of this motif.

In addition to $P\sigma^N$, a σ^{70}-type promoter sequence lies between *mmoX* and *mmoY*. Primer extension and Northern blotting experiments performed by Nielsen *et al.,* (1997), suggested that there may be a transcriptional start site corresponding to this promoter and that transcription from this promoter is copper-sensitive. Western blots performed using sMMO-hydroxylase specific antisera to probe the expression of the sMMO-hydroxylase proteins in cell extracts of the Gm1 and JS1 strains revealed that whilst the α-subunit of the hydroxylase was absent, the β-subunit was present, indicating that this promoter may be active. However, RT-PCR studies examining transcription from this promoter in these strains must be conducted to confirm its activity. This could be achieved by designing *mmoY*-specific primers for RT-PCR assessment of *mmoY* transcription. These primers could then be used to assess the presence of *mmoY* transcripts in RNA extracted from the Gm1 and JS1 strains, where

mmoX transcription is disabled. Therefore any transcripts detected in these strains would originate from the promoter 5' of *mmoY*.

At the same time, the transcription of the two *pmo* operons in *Methylosinus trichosporium* OB3b is known to be repressed at low copper to biomass ratios (Nielsen *et al.*, 1997). An alignment of the promoter region of the two copies of the *pmo* operon of *Methylosinus trichosporium* OB3b reveals divergence 4 bp 5' of the -35 region of the pmo_1 promoter. This implies the involvement of a repressor protein as activators rarely bind to DNA overlapping the -10 region of σ^{70} promoters (Collado-Vides, 1991). In this model, an unknown repressor binds in the *pmo* promoter region and inhibits transcription at low copper concentrations. However, data presented in chapter 3 of this thesis and by Stolyar *et al.*, (2001) would suggest that this repression is incomplete. It is also clear that transcription of the *pmo* operon of *Methylosinus trichosporium* OB3b occurs from σ^{70} promoters located 5' of *pmoC*. The presence of two promoter sequences in the 5' region of copy 2 (but not copy 1) also raises the possibility of differential expression of the two copies of *pmo* in this organism, as has been observed recently in *Methylococcus capsulatus* Bath (Stolyar *et al.*, 2001).

Upon the addition of copper to the cells, transcription of the sMMO operon is completely abolished, whilst transcription of the *pmo* operon is derepressed (Nielsen *et al.*, 1997). In the presence of copper, σ^N is unable to form an open transcription complex at $P\sigma^N$, as MmoR is inactive and thus unable to promote open complex formation. Conversely, the presence of copper releases the hypothetical *pmo* repressor and results in maximal expression of the *pmo* operon from one or both of its promoters. However, evidence supporting this part of the model is scarce and so remains highly hypothetical.

8.3 Future Prospects

The work presented in this thesis has greatly improved the current understanding of the 'copper switch'. However, several questions must be answered if we are to more fully understand this complex regulatory system. Firstly, what is the nature of the signal which is relayed to MmoR and the unknown repressor of the *pmo* operon? Is it copper ions themselves or is there a central copper sensing system which inactivates both proteins in response to copper? One clue regarding copper-binding to MmoR was provided by the work of Lloyd *et al.,* (1999) (see section 1.4.3) who showed that a strain of *M. trichosporium* OB3b containing the *mmo* cluster (including the *mmoR* and $groEL_{mmo}$) expressed sMMO at elevated copper concentrations, when compared to the wild-type. In light of the discovery of the role played by MmoR in *mmo* transcription, it is possible that the decreased sensitivity of this strain was due to multiple copies of the MmoR protein, which, if it binds copper directly, requires a higher concentration of copper to inactivate all the MmoR polypeptides within the cell. It is also clear that the *pmo* and *mmo* operons are not the only gene clusters whose transcription is affected by copper levels, since extensive internal membrane systems are constructed within the cells in response to increased copper levels (Scott *et al.,* 1981) and several protein spots were found to be differentially expressed in response to copper in the proteomic study described in Chapter 7. It is also possible that other environmental factors such as carbon source (Finch, PhD thesis, 1997; Chapter 4) and oxygen levels (Kim *et al.,* 2001) influence the expression levels of these gene clusters and add further layers of complexity to the model illustrated in figure 8.2. In order to accurately define the response of the *pmo* and *mmo* clusters to carbon source and oxygen levels it will be necessary to produce stable chromosomal reporter gene fusions in a similar manner to that reported by Stolyar *et al.,* (2001) (discussed in chapter 3).

The work in this thesis has chiefly focussed upon *Methylosinus trichosporium* OB3b and the proposed model refers to experimental evidence gained from this organism. The *mmo* operons from several methanotrophs have now been cloned and sequenced, revealing striking similarities in the promoter regions 5' of *mmoX* (McDonald *et al.,* 1998; Shigematsu *et al.,* 1999 ; Nielsen *et al.,* 1996). This suggests that the model involving an EBP and σ^N may be widespread among methanotrophs. Further evidence for this was provided by the demonstration of copper-regulated

expression of the *mmo* operon from *Methylosinus trichosporium* OB3b in the pMMO-only methanotroph *Methylomicrobium album* BG8 (Lloyd *et al.*, 1999). The expression vector used for expression in *Methylomicrobium album* BG8 contained the *mmoR* gene and it may be that *mmoR* was functional in this organism and that *Methylomicrobium album* BG8 contained the copper-sensing and relay mechanisms to allow activation and inactivation of MmoR.

Despite the observations made in *Methylomicrobium album* BG8, expression of sMMO in non-methanotrophic bacteria, such as *E. coli* and *Pseudomonas putida* has been unsuccessful for other reasons. The hydroxylase polypeptides are produced in an inactive form, indicating that although transcribed in these organisms assembly of a functional protein was not achieved (Jahng and Wood, 1994; Lloyd, 1997). If, as it is tempting to speculate, the $groEL_{mmo}$ gene product is an sMMO specific chaperone, then it may not be functional in these hosts. GroEL proteins require the product of another gene, *groES*, with which they are often co-expressed (Lund, 2000) and it may be that the $GroEL_{mmo}$ is unable to interact with the GroES proteins from *E. coli* and *Pseudomonas putida*. As mentioned above, Lloyd *et al.*, (1999b) achieved expression of the sMMO enzyme in the pMMO-only methanotroph *Methylomicrobium album* BG8. Thus, another explanation of the success of this experiment may be that the GroES from *Methylomicrobium album* BG8 is effective as a co-factor for $GroEL_{mmo}$ from *M. trichosporium* OB3b, thus allowing functional sMMO expression. Future work must focus on determining the function of this chaperone in *Methylosinus trichosporium* OB3b by the construction of a marker exchange mutant in the $groEL_{mmo}$ gene, and the subsequent analysis of its phenotype with respect to sMMO expression. It may also be possible to achieve expression of sMMO in *E. coli* by expressing a methanotroph derived GroES protein, preferably from *Methylosinus trichosporium* OB3b, in a strain also containing a *M. trichosporium* OB3b sMMO expression vector.

As discussed above, there may be additional factors involved in both the regulation of the *pmo* and *mmo* operons and assembly of the functional sMMO complex. There are several possibilities for discovering such additional factors. The close proximity of the *mmoR* gene to the *mmo* cluster indicates that additional regulatory factors may be located 5'of the *mmoR* gene, cloning and mutational analysis of this region may yield information regarding further genes which may be involved with the copper switch. The preliminary proteomic study presented in

chapter 7 has confirmed that several gene products are differentially expressed in response to copper. However, the imminent arrival of the *Methylococcus capsulatus* Bath genome sequence data and the use of proteomic techniques for this organism represent the best chance of identifying additional factors involved with the copper switch.

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APPENDIX A:

Nucleotide sequence information for *rpoN* gene cluster from *Methylosinus trichosporium* OB3b

```
LOCUS       rpoN cluster      4329 bp    DNA
DEFINITION  rpoN gene cluster from Methylosinus trichosporium OB3b (pGPS519)
ACCESSION   OB3bclus
KEYWORDS    Methylosinus trichosporium OB3b
SOURCE      Graham Stafford.
  ORGANISM  Methylosinus trichosporium OB3b

REFERENCE   1  (bases 1 to 4329)
  AUTHORS   Graham Stafford
  JOURNAL   Unpublished.
FEATURES             Location/Qualifiers
     CDS             269..568
                     /gene="ORFR"
                     /product="part of putative response regulator"
     CDS             753..1031
                     /gene="ORF3"
                     /product="possible orf: conserved hypothetical protein with
homology to conidiation proteins"
     CDS             complement (992..1552)
                     /gene="ORF2"
                     /product="phosphotransferase enzyme 2"
     CDS             complement (1806..2384)
                     /gene="ORF1"
                     /product="sigma 54 modulation protein"
     CDS             complement (2761..4033)
                     /gene="rpoN"
BASE COUNT      818 a   1342 c   1379 g    787 t
ORIGIN
        1 AVCGAAGTCA GTAAGCAAGA AAAGCGGAAG AGCGCCCAAT ASGCAAACCG CCTCTCCCCA
       61 GCGGTGTTGG CAGATTCATT AATGCAGCTG GCAAACAGGG TTTCCCGACT TGAAAAGCGG
      121 GCAGTGAGCG CAACCCAATT AATGTGAGTT AGCTCACTCA TTAGGCACCC CAGGCTTTAC
      181 ACTTTATGCT TCCGGCTCGT ATGTTGTGTG GAATTGTGAG CGGATAACAG TTTCACACAG
      241 RAAGCAGTTA TGACATGATT ACGAATTCGA GCTCGGCCAT CGCGTCGTCG CCGTGGCCCG
      301 CACACATAAA GAAGCCGTGG CCGCGATCGC CAAGACGCCG CCCGGTTTGA TCCTGGCCGA
      361 CATCCAGCTG GCCGACAACA GCTCAGGTGT GGAAGCGGTT AACGAAATCC TCGGCTCGGT
      421 GTCGACGCCG GTGATCTTCG TCACCGCCTA TCCGGAACGC TTCCTCAAAG GCGAGCCGCC
      481 GGAGCCCGCT TTTCTGATCG CCAAGCCATT CAGCGTAGAC AGCCTCAAGG CGATCATCAG
      541 CCAAGCTTTA TTTTTCGACA GACGCTCCCA TGTCAAAGGC AATGATAAGC ACAACTGATC
      601 GTATAAAGCG ATAGCTTAAG TAGTCGTTTG TTTTCGTACG TATAGACCAC AGGTCAAGTT
      661 TGGCCAAAAA AAACTTCTAT CCTGCGCCAA GAGAGCGCAA CCTTGTCTTC ACTCGGGCGT
      721 TGACTCCCGG GGGACAGGAG AAGGCAGATC AAATGACCGC TATGCAGGAA CCCAAGAAGT
      781 CGAACCGGGG CTTTGCGTCG ATGGACCCCG AGAAGCAGCG CGCCATCGCC CGCAAGGGCG
      841 GCCAGAACGT CCCCGACGAG AAGCGCAGCT TCTCTCAGAA TCCGGAACTG GCGGCGAAAG
      901 CCGGGCGCAA AGGGGGGCAG AGCGTCGATC CGACCAAGCG CAGCTTCTCC CGCGACCATA
      961 CCCTCGCGTC CGAAGCCGGG CGGAAGGGCG GTCACGCGTC CCATTCCAAG CCGAGAACAG
     1021 CCGCGGAGTA ATCCGCGGCC GCCCGCGCGC GCCTTCGGAA GCGATTCGGC GCGAGAGCGC
     1081 CGAGCGCCTC AGCGGCGTGT GGCTTGGTGG TTTGCGCGAT GATGGAATAG AGAGCCGACG
     1141 GATCGCGGGT CGTGCGAATG GTCGCGACCA CGGCCGGATC GCGCAGCATG CGGGCGGCGC
     1201 AAGCGAGCGC TTTCAAATGA TCGGCCCCCG ATGATTCGGG GGCGATCAGC AGAAAAACCA
     1261 GATCGACCGG CGCGCCGTCC AGCGCGTCGA AATCGATCGG GCGCTCGAGC CGCGCGAAAA
     1321 TCCCGAAGAT CGATTTGACG CGCGCCAGCT TGCCGTGCGG AATGGCGATG CCATTGCCGA
     1381 TGCCGGTCGA GCCGAGACGC TCGCGCTGCA ACAGTGCGTC GAAGATTTCG CGGGCCGGCA
     1441 GGCCGCAAAT TTCCGCAGCC TTCTCGCTCA GCTCCTGAAG CGCCTGCTTC TTGGTGTTGG
     1501 ACTTGAGCGT CGGAATAATC GCTTCCGATG TGAGCAGATC AGCGAGCCGC ATGAGGCGTC
     1561 AATCCCATTC ACGATCTGAT TTCGGCATGA GAACGCGCCG TCGCCGGGGT CCGGTGAAGG
     1621 CTCATTCGAA GCTATTCGCC TGAACGCCGG CGCGAGGCGA CGAGGTTTGC GTCGCGGAAA
     1681 CTCCGTGGCC GTCGCGCTGA ACGACTCTGC GCCGCATGAT ATGGCGCCAC TGGAGCTTCC
     1741 GCATTCTCAT CGCCGCCGCT TCCCATTCCA TGGGCCGCCC CGTTCTATGG CAAAATATAT
     1801 GCCGCCTATT CTCCGCCGGG CGCGTCGATC CATCCGATAT GGTCGTCGCT GCGCCGGTAG
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1861 ACAACATTGA GGCGGCCGGT ATCCGCGTGC CGGAACACGA CCACGGGCGC GCCGGTCAGG
1921 TCGAGATCGA GCACCGCCGC CGACACGGTG AGCCGGCGCA GGCGCGCGGT CGACTCGGCG
1981 ACGATCGTCG GCGCGAATTC CTTGGGCGCC TCGACCTCCT GATCGGGCGC CTCGAGCACA
2041 TAGCTGGCGG CGACCTCGCC GAGGTCGGGG CCGGCCGCGG CGTGCTCGGC GCGATGCTCC
2101 TTCAGCCGGC TCTTGTAGCG GCGCAGCCGT TTCTCGATGC GGTCGGCCGC GAGGTCGAAA
2161 CAGGCGTAGG GCTCTTGAGC GCGGCCATCG GCCTGCAGCA CGATGCCGGA GGTCAAATGC
2221 AGGCTGCAAT CGGCGCGATA GCCCGATCCT TCCGGCGCGA TCGTCACATG GCCGCTGATC
2281 TCGCCGTCGA AATATTTGGA GGCCGCGGCG CCGAGCCGTT CGGCGACATG GGCCCGCAGG
2341 GACTCGCCGA TATCGATATT CTTTCCGGAC ACCCGCAAAG ACATGCTTGC TCCACCTCAT
2401 CGAGCCGCGC GCTCGCGCCG CGAGGGCGCG CGCCGCTGAC GGAAATGTCC TGAATGCGGG
2461 CTCTTCGCCA TTCGCGCCCG GGCAGCCCGC TCCGCCCGAA TGACGCTCGC CCCTCAACTC
2521 GTCCTTCTGC CGCGCGAACA TGGCGGAAAT TCGGGCGCCT GCCCAACCAT TGATCGGAAG
2581 CGTTAAGTTG CTGGGCGCTC GAAGTCAATG GCCGATCGAG CGCCGAGGCC TGCTCCAGCA
2641 TTCCTTCTTT TGAGCGAGTT CCGATCGACC GAACGACGCG TCGAACGGAA AGCGCTCTAG
2701 AATTCTTTCG TTTGAGCGAA TTCTGATCGA TCGAACGATT CCGTTCGATC GGAAAGCGCT
2761 CTAGAGAGCC GGTTGAACGG TCTCGCGCAG CTGGCTCTGC TTGGCCCTGC GGCGGTCCAC
2821 CGATGACGGA ATGCGCAGGC TGTCGCGATA TTTGGCGACA GTGCGCCGGG CGATGTCGAT
2881 ATCGATCTGC TTGAGCCTCG CGACGATGGC GTCGTCCGAC AGCACGTCGA AGGGGCTCTC
2941 CTTGTCGATC ATCTGCTTGA TCTTGTAGCG CACCGCCTCG GCCGAATGGG CCTCGCCGCC
3001 ACTGGTGGTG GCGATGGAGG CGGAGAAGAA ATATTTCAGC TCGAAAATTC CCCGCGGGGT
3061 CATCATATAT TTGTTCGAGG TGACGCGCGA GACTGTGGAT TCGTGCATGC CTATGGCGTC
3121 GGCGATGGTG CGCAGATTGA GCGGCCGCAG ATGCTCGACG CCCCTGGCGA GAAAAGCGTC
3181 CTGCAGGCGC ACGATCTCGG AGGCGACCTT GAGAATGGTG CGCGAGCGCT GCTCGAGGCT
3241 CTTGGTCAGC CAGTTGGCGT TCTGCAGGCA GCTCGAGATG AAGGTCTTGT CGCCGTCGCG
3301 CTTGGCGCCG GCGCTCACCT GCGCTGCATA GGAGTGATTG ACCAGCACGC GCGGCAGCGC
3361 ATCCGAGTTC AATTCGACGA TCCAGGAGCC GTCGGCGGCC GGCCGCACGA TCACATCGGC
3421 GACGAGCGGC TGGAGCGGCG CGCCGCCGAA GGCGCGGCCC GGCTTGGGAT CGAGCCGACG
3481 CACCTCGGCC GCCATATCGG CGATGTCCTC GTCGTCGACG CCGCAGAGGC GCGCGAGCTG
3541 GGCGAAGTCG CGCTTGGCGA GGAGCGGCAG ATTGGCGATG AAGATCTGCA TCGCCGGATC
3601 CAGCCGGTCG CGCTCGCGCA GCTGCAGAGC GAGGCATTCT GAGAGATCGC GGGCCCCGAC
3661 GCCGGAGGGG TCGAAGCGCT GAATGATCGC CAGCACGGCT GCGACCGCAG CCGGATCGGC
3721 GTCGAGCCGC GCCGCGATCT CGGCGACGTC CTCGCGCAGA TAGCCGACCT CGTCGATGGC
3781 GTCGATGATC GCGTGGCCGA TGATGCGATC GATCGGATCG GCGCAGGCGA GCGCCAGCTG
3841 CTCGCCGAGA TAATCGTGAA GGCTCGCCTG AGAGGCGACA TAGGCTTCGA GATTGGGAGC
3901 CTCGCCGTCG GCGGGCGCGC CGCCGGCGCC GTTCCAGGAG CTGGCCGAAA GGCCGGCGCC
3961 CTCGAGCGCG CCCATCGAAT CGGCCGGGCT GCGCGCATTG TCCAGCTCGA AGGCGTTGCC
4021 GATCTCGGTG CCGAGCTCGG TACCCGGGGA TCCTCTAGAG TCGACCTGCA GGCATGCAAG
4081 CTTGGCACTG GCCGTCGTTT TACAACGTCG TGACTGGGAA AACCCTGGCG TTACCCAACT
4141 TAATCGCCTT GCAGCACATC CCCCTTTCGC CAGCTGGCGT AATAGCGAAG AGGCCCGCAC
4201 CGATCGCCCT TCCAACAGTT GCGCAGCCTG AATGGCGAAT GGCGCCTGAT GCGGTATTTT
4261 CTCCTTACGC ATCTGTGCGG TATTTACACC GCATATGGTG CACTCTTAGT ACAATCTGCT
4321 CTGATGCCG

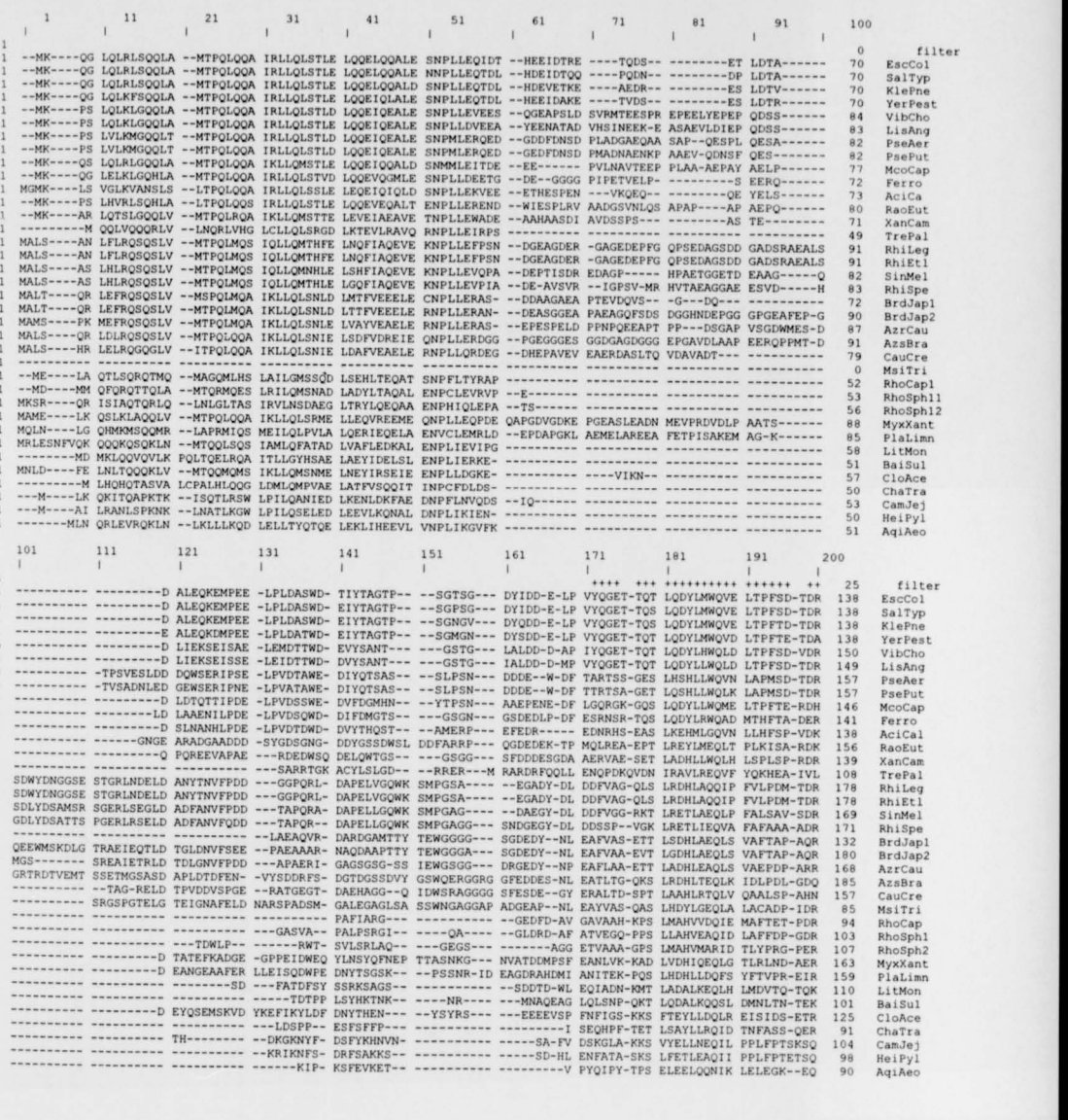
APPENDIX B:

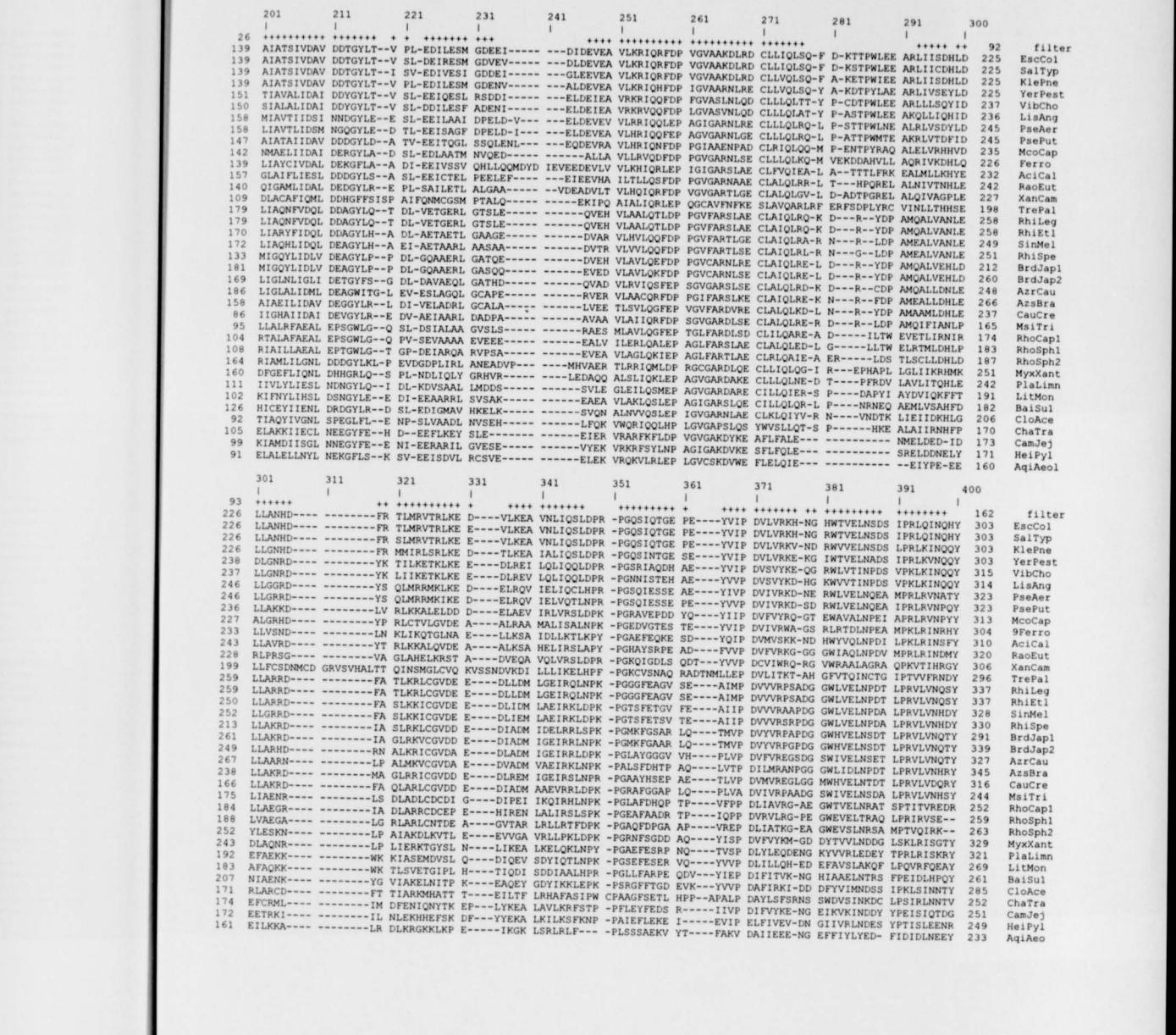
Amino acid alignment of derived amino acid sequence from 30 *rpoN* genes

This alignment was compiled using ClustalW based on 307 amino acids (171-616). Amino acid positions included in constructionof figure 5.6 are indicated (+) in the row labelled filter. Acession numbers are shown in figure 5.6.

Key to organisms:

Esccol: *Escherichia coli*
SalTy: *Salmonella typhimurium*
KlePne: *Klebsiella pneumoniae*
YerPest: *Yersinia pestis*
VibCho: *Vibrio cholerae*
LisAng: *Listonella anguillarum*
PseAer: *Pseudomanas aeruginosa*
McoCap: *Methylococus capsulatus* Bath
Ferro: *Thiobacillus ferooxidans*
AciCal: *Acinetobacter calcoaceticus*
RaoEut: *Ralstonia eutrophus*
XanCam: *Xanthomonas campestris*
TrePal: *Treponema pallidum*
RhiLeg: *Rhizobium leguminosarum*
RhiEt: *Rhizobium etli*
SinMel: *Sinorhizobium meliloti*
RhiSph: *Rhizobium sp.* NGR234
BrdJap1: *Bradyrhizobium japonicum* copy 1
BrdJap2: *Bradyrhizobium japonicum* copy 2
AzrCau: *Azorhizobium caulidonans*
AzsBra: *Azospirillum brasilense*
CauCre: *Caulobacter crescentus*
MsiTri: *Methylosinus trichosporium* OB3b
RhoCap: *Rhodobacter capsulatus*
RhoSph1: *Rhodobacter sphaeroides* copy 1
RhoSph2: *Rhodobacter sphaeroides* copy 2
MyxXan: *Myxoxoccus xanthus*
PlaLim: *Planctomyces limnophilus*
LitMon: *Listeria monocytogenes*
BaiSub: *Bacillus subtilis*
CloAce: *Clostridium acetobutylicum*
ChaTra: *Chlamydomonas trachomatis*
CamJej: *Campylobacter jejuni*
HeiPyl: *Helicobacter pylori*
AqiAeo: *Aquifex aeolicus*





```
     401        411        421        431        441        451        461        471        481        491        500
      |          |          |          |          |          |          |          |          |          |          |
163   +++          ++++  +++++++++ ++++++++++ ++++++++++ ++++++ +++  ++++++++++ ++++++++++ ++++++++++ ++ +++++++  245   filter
304  ASMCNNAR-- NDGDSQFIRS NLQDAKWLIK SLESRNDTLL RVSRCIVEQQ QAFFEQGEEY MKPMVLADIA QAVEMHESTI SRVTTQKYLH SP-RGIFELK  400   EscCol
304  AAMCNSAR-- NDADSQFIRS NLQDAKWLIK SLESRNDTLL RVSRCIVEQQ QAFFEQGEEY MKPMVLADIA QAVEMHESTI SRVTTQKYLH SP-RGIFELK  400   SalTyp
304  AAMGNSTR-- NDADGQFIRS NLQEARWLIK SLESRNDTLL RVSRCIVEQQ QAFFEQGEEF MKPMVLADIA QAVEMHESTI SRVTTQKYLH SP-RGIFELK  400   KlePne
304  AAMGNSVR-- NDSDGQFIRS NLQEAKWLIK SLESRNETLL KVARCIVEQQ VEFFEKGAEF MKPMVLADIA QAVDMHESTI SRVTTQKFLH SP-RGIFELK  400   YerPest
316  ADLMRG---- NNAESNYIRT NLQEAKWLIK SLESRNETLL KVAKCIVEHH HDFFEYGEEA MKPMVSTDVA MAVEMHESTI SRVTTQKYMH TP-RGIFELK  410   VibCho
315  AALGKG---- NSADSNYIRS NLQEAKWLIK SLESRNETLL KVAKCIVEHQ RDFFEYGEEA MKPMVLNDVA LAVEMHESTI SRVTTQKYMH TP-RGIFELK  400   LisAng
324  AGMVRRAD-- SSADNTFMRN QLQEARWFIK SLQSRNETLM KVATQIVEHQ RGFLDYGEEA MKPLVLHDIA EAVGMHESTI SRVTTQKYMH TP-RGIFELK  409   LisAng
324  AGFVRRAD-- TSADNTFMRN QLQEARWFIK SLQSRNETLM KVATRIVEHQ RGFLDHGDEA MKPLVLHDIA EAVRMHESTI SRVTTQKYMH TP-RGIFELK  420   PseAer
314  SGLIRRAD-- SSSDNVTMRN HLQEARWFIK SLQSRNETLL KVARAIVDRQ REFLEIGETA MKPLVLRDIA EEVSMHESTI SRVTTQKYMH TP-NGIYEFK  410   PsePut
305  ADMAGG---- KDAAHKYIQD QLNEARWFIK SLQSRQDTIL KVARAIVERQ KDFFANGPES MRPMVLRHIA DAVEMHESTV SRVTNQKYMI TP-RGLYEFK  399   McoCap
311  SGMIKRAD-- QSDDNQYLRN QMLEAKNFIK SVDERHKTLL KVASCIVQHQ REFLEIGAEG MKPLVLRDVA EEVELHESTV SRVTTNKFLL TP-RGLFELK  407   Ferro
321  AQILRGAK-- GESGTAGLQQ KLQEARWLIK NIQQRFDKIL RVSQAIVERQ KNFFSHGEIA MRPLVLREIA DTLGLHESTI SRVTTNKYMA TP-MGTFELK  417   AciCal
307  ENLIRSCG-- -ESDAGYLRG QLQEARWLLK SLEARGETLL RVVRCLLEHQ AGFLEFGAQA LRPLTLREIA GELGLHESTI SRAIARKYVR TP-RGTIALR  402   RaoEut
297  CMHSKA---- -AEKNHALKA CMHDAI SLVS MLSYRERTLL DIAKTIVHYQ CGFFDHGPAK LTPLRMTDVA HRTGLSVSTV SRIVRDKWLQ YG-SQHFSLR  390   XanCam
338  FSRVTR---- NGEDHAFLSE CLQSANWLTR TLDQRAKTIM KVASEIVRQQ DAFLLNGVDH LRPLNLKTVA EAIKMHESTV SRVTSNKYML TP-RGLFELK  432   TrePal
338  FSRVTR---- NGEDHAFLSE CLQSANWLTR TLDQRAKTIM KVASEIVRQQ DAFLLNGVDH LRPLNLKTVA EAIKMHESTV SRVTSNKYML TP-RGLFELK  432   RhiLeg
329  FTEISRSSRK NSGEQAFLNE CLQNANWLTR SLDQRARTIM KVASEIVRQQ DAFLIHGVGH LRPLNLRIVA DAIKMHESTV SRVTSNKYML TP-RGLFELK  427   SinMel
331  FAEISRHSQK NSAEQAFLSE CMQNANWLTR SLDQRARTIL KVASEIVRQQ DAFLVHGVDH LRPLNLRIVA DAIKMHESTV SRVTSNKYRL TP-RGLFELK  429   RhiSpe
292  YSKLSKKIG- KDVDKSYFND ALQNATWLVR ALDQRARTIL KVATEIVRQQ DGFFTLGVAH LRPLNLKAVA EAIQMHESTV SRVTANKYMA TN-RGTFELK  389   BrdJap1
340  YSELSKKIG- KDGDKSYFTD ALQNATWLVR ALDQRARTIL KVATEIVRQQ DGFFTHGVAH LRPLNLKAVA DAIQMHESTV SRVTANKYMA TN-RGTFELK  437   BrdJap2
328  HATVAKAAR- SAEEKTFLAD CLQSASWLTR SLDQRARTIL KVASEIVRQQ DAFLVHGVRH LRPLNLRTVA DAIGMHESTV SRVTSNKYIS TP-RGVLEMK  425   AzrCau
346  FARISGTAR- NKADKEYITE RFQSANWLVK SLHQRATTIL KVASEIIRQQ DAFFIHGVSH LKPLILRDIA EAIGMHESTV SRVTTNKFMA TP-RGVFELK  443   AzsBra
317  HARVSKGAR- SDQEKTFVAD CMASANWLVK SLDQRAKTIL KVASEIVRQQ DGFLAFGVEH LRPLNLKTVA DAIGMHESTV SRVTSNKYIA TP-RGVFELK  414   CauCre
245  AAQVSAGAK- RDGDKTFISS CLQNANWLTK SLEQRSRTIL KVASEIVRLQ DAFLARGVEH LRPLNLRTIA DAIGMHESTV SRVTSNKYMM TP-RGIFELK  342   MsiTri
253  FADGTA---- DAKARAERRR -RGRGPGAGE ALERRRDTDL RTAAVLVARQ SAFLDKGPAH LVPLTLEDVA SELGLHASTI SRAVSGRMIQ TQ-TRALPLR  346   RhoCap
260  -AGDTG---- DRQADAWLAR ARSQARWLER AVERRQATLL RTAVCLVRHQ ADFLDQGPRA LRPLSMEEVA LELDLHPSTI SRATATRLIE TP-RGLIPLR  353   RhoSph1
264  -PDKR----- --PTTPAARA AWTQAQAVGR MIENRNATLL RVAREILARQ EAALDEGPSA LVALTMTEVA EALGIHESTV SRVVAGTCVD TP-RGTWWLR  354   RhoSph2
330  RNALKTGAVG PGQTKDFIQD KLRSAMWLIR SIHQRQRTIY KVTESIVKFQ RDFLDKGIAY LKPLILRDVA EDIGMHESTV SRVTTSKYVH TP-QGIFELK  428   MyxXant
322  IEMMRQGAS- DPTTREYIKR KVESAKWLIE SIEQRHNTLK RVAQAIVDHQ IEFLEKGPEF /SPLKMQQIA DIVKVHVTTV SRAVDDKWIQ TP-RGLFPLK  419   PlaLimn
270  YETMRATE-- EKDVAQFLRE KAGEFDWIKK GIEQRESTLQ RVGEAIVSHQ KAYFLDNSAH LQPLTLKEVA EELGVHESTV SRAVNGKYME TT-NGVYELK  366   LitMon
262  RTLLSSGS-- CQDTVSYLSA KYQEWRWLSR ALRQRKQTIT RIINELITRQ KDFFLKGRSA MKPLTLREVA DCLSLHESTV SRAIKGKTIQ TP-YGLFEMK  358   BaiSul
286  KNILNTEN-- DKKDVDYVKE KLNSAMFLLK SIEQRKTTLY EVLNEILRYQ KAYFVGKTNY LRPMTLKDIS EKLGMHESTI SRAIREKYVY IDTKGLVKIR  383   CloAce
253  LDIYPSLP-- -REEKDHLSQ QIRAAKQLLR NVKKREETLL AILRVLIPYQ EEFLLKKRTS PKAFSVKQIA RELSLHEATV CRAIDNKTLA TP-VGLLPMR  348   ChaTra
252  LEHD------ ------FLSH YIKEAKNLVD ALAMRKATLY KIGLMIVEYQ YDFFM-GKE- IKPMTFKDLA LDLERNASTI SRAVANKYLS CE-RGLIPLR  336   CamJej
250  FKDSG----- ------YLKE KLKEAKDLID ALNLRKATIY KIGLMLLEYQ YDFFK-GKE- LRPLKLLDLA NEFNHSVSTI SRAISNKYLA CE-RGVFPIK  335   HeiPyl
234  WELYKK---- SRNLQKELKE AFERYESIRK VLDIRRRNLR KVLEKIVERQ KDFLT-GKGS LKPLTLREVS SEIGIHESTL SRIVNSKYVK TP-VGTYSLR  327   AqiAeo

     501        511        521        531        541        551        561        571        581        591        600
      |          |          |          |          |          |          |          |          |          |          |
246  ++++++++    +          ++  +++ +++++  ++++    ++ ++++++++++ +++++ ++++ ++++++++++ +++++++                    307   filter
401  YFFSSHVNTE -GG------G E------ASS TAIR-ALVKK LIAAE-NPAK PLSDSKLTSL LSEQG-IMVA RRTVAKYRES LSIPPSNQRK QLV-------  477   EscCol
401  YFFSSHVNTE -GG------G E------ASS TAIR-ALVKK LIAAE-NPAK PLSDSKLTSL LSEQG-IMVA RRTVAKYRES LSIPPSNQRK QLV-------  477   SalTyp
401  YFFSSHVNTD -SG------G E------ASS TAIR-ALVKK LVAAE-NPAK PLSDSKLTTL LCEQG-IMVA RRTVAKYRES LSIPPSNQRK QLV-------  477   KlePne
411  YFFSSHVSTD -NG------G E------CSS TAIR-ALIKK LVAAE-NPAK PLSDSKIATL LADQG-IQVA RRTIAKYRES LGIAPSSQRK RLL-------  487   YerPest
410  YFFSSHVSTD -SG------G E------CSS TAIR-ALIKK LVAAE-NTAK PLSDSKIADL LADQG-IQVA RRTIAKYRES LGIAPSSQRK RLL-------  486   VibCho
421  YFFSSHVSTA -EG------G E------CSS TAIR-AIIKK LVAAE-NAKK PLSDSKIAGL LEAQG-IQVA RRTVAKYRES LGIAPSSERK RLV-------  497   LisAng
421  YFFSSHVSTS -EG------G E------CSS TAIR-AIIKN LVAAE-NQKK PLSDSKIAGL LEGQG-IQMA RRTVAKYRES LGIAPSSERK RLM-------  497   PseAer
411  YFFSSHVSTD -SG------G E------CSA TAIK-AFLKE IVSKE-DATR PLSDHAIAGM LKDKG-INVA RRTIAKYREA MGIPPSNERK QLF-------  487   PsePut
400  YFFSSHVGTD -SG------G S------ASA TAIR-ALLIK MTQAE-DAQH PLSDAEIARV LADQG-IQIA RRTVAKYREA ANVPPASQRR RL--------  475   McoCap
408  YFFSSHVGTT -SG------G E------ASS TAIR-AKIKK LVADE-NPRK PLSDNTIANL LKEEG-IDVA RRTVAKYRES LHIPSSSDRK VLI-------  484   Ferro
418  YFFGSHVSTE -TG------G A------ASS TAIR-ALIKQ LIGAE-DPRN PLSDSRIAEL LGEQG-FVVA RRTVAKYREA LKIPAVNLRK SL--------  493   AciCal
403  AFFASGIDTD -SG------G E------ASS TAIQ-AMIRR LIDAE-NPRK PLSDAKLADL LKTSG-VPVA RRTVAKYREA MNISASHERV RIA-------  479   RaoEut
391  YFFSPRVLST EEYRDRSSLG QNYPSSPHSK VSVK-YRISR LIQEVRTQRI SLSDRRIAQL LGEQG-IKCA RRTVNKYRSE LRTCSSS--- ----------  475   XanCam
433  YFFTVSISAV -EG------G EQ-----HSA EAVRATRFRA LIMQE-SPDA VLSDDDIVDM LKKGG-IDLA RRTVAKYREA MNIASSVQRR REKRAL----  514   TrePal
433  YFFTVSISAV -EG------G EQ-----HSA EAVRATRFRA LIMQE-SPDA VLSDDDIVDM LKKGG-IDLA RRTVAKYREA MNIASSVQRR REKRAL----  514   RhiLeg
428  YFFTVSIGSA -EN------G DA-----HSA ESVR-HRIRT MINQE-SADA VLSDDDIVDV LKQAG-VDIA RRTVAKYREA MSIPSSVQRR REKRAL----  508   SinMel
430  YFFTVSIGSA -EN------G DA-----HSA ESVR-HRIRT MISQE-SADA VLSDDDIVDI LKRAG-VDIA RRTVAKYREA MNIPSSVQRR REKRALPRQP  514   RhiSpe
390  YFFTASIPSA -DG------G EA-----HSA EAVR-HRIKQ LIESE-EPSA VLSDDAIVER LRVSG-IDIA RRTVAKYREA MRIRSSVQRR RDNMWSTMNS  474   BrdJap1
438  YFFTASIASA -DG------G EA-----HSA EAVR-HHIKQ LIDSE-APAA ILSDDTIVER LRASG-IDIA RRTVAKYREA MRIPSSVQRR RDKQSALGNV  522   BrdJap2
444  YFFTSAIQGA -DG------Q AA-----HSA EAVR-YRIKA MIDAE-KPDD VLSDDKIVEI LRGEG-IDIA RRTVAKYREA MRIPSSVQRR REKQ-ALRSD  509   AzrCau1
415  FFFTSAIQSS -EG------G EA-----HSA ASVR-HKIKG LVDAEKCEAD VHSDDRIVEI LKAAG-VDIA RRTVAKYREA MRIPSSVERR RLMKEAV---  497   AzsBra
343  YFFSASIATT -SG------G EA-----HSA EAVR-YKIKQ MIDKE-SPFD VLSDDAIVAR LKQID-IDIA RRTVAKYRDS LRIPSSVDRR RAKQSQLRET  427   MsiTri
347  AFFSRAVSTQ -GG------G EA-----VSR DSLD-FVQRT WAAK--IRQN PLSDDAIVTL AERAG-LRIA RRTVAKYRST LGLASSYERR RAAAAR----  426   RhoCap
354  AFFSRSVSSD -GP------E AP-----QSQ DAIM-ALVRE IIARE-DRTK PFSDDAIVKQ AKLAG-AVLA RRTVTKYRET LGIPSSYDRK RAAAAA----  434   RhoSph1
355  RMFSGRLA-- -EG------G P-------SA AAIR-AAIAR LVAQE-DPAA PLSDGALVEA LAAED-MQLA RRTVAKYREM LNIPPGHRRR RRPSRSA---  432   RhoSph2
429  YFFNSSIARV -SG------E D------TAS EAVK-HHIKQ LVAQE-DARN PYSDQKIVEL LRSQG-TEIA RRTVAKYREV LGILPSSKRK RYY-------  505   MyxXant
420  RFFGGGTTTA -EG------E E------VAW HIIR-LKLKE IVDGE-NKDD PLSDDALVEE LAKHG-FNLA RRTVTKYRKA LNIPSSRQRR SY--------  495   PlaLimn
367  RFFASGLQKK SSE------N ENIGD--ISS TTIK-KLVQE FVAAE-DKLR PLSDQKIVDM LAEKE-IQVS RRAIAKYRLE LNIPSSSKRK RF--------  447   LitMon
359  LFFSAKAEAS -GD------G D------ASN YAVK-THLEN LINQE-DKTK PLSDQKLVDL LYEQHGIQIS RRTVAKYRDQ MNIPSSAARK RYK-------  436   BaiSul
349  SLFPQAVGSC PDQ------- --------SK ATIL-HWIHQ WISTE---KH PLSDAAISQK IIEKG-IPCA RRTVAKYRSQ LNILPAHQRK HLCSVLTTTR  428   CloAce
337  DFFAFALD-E -EG------- -------ETS NVGVKEFVAN LVKNE-DRNK PLSDSKILEL IKEEFKVDIG RRTITKYRKH LNIASSTDRK KLYELEG---  416   ChaTra
336  HFFSIALD-N -S------- -------ETS NAVIKDYLLE LIKNE-DKKE PLSDAKILEL IEEKFHLKMV RRTITKYRQL LNIASSSERK RLYLMRA---  414   CamJej
328  TFFVRESA-- -EG------- -------LTQ GELM-KLIKE IVENE-DKRK PYSDQEIANI LKEKG-FKVA RRTVAKYREM LGIPSSRERR I---------  398   AqiAeo
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   601        611
    |          |
308
478 ---------- ----------
478 ---------- ----------
478 ---------- ----------
478 ---------- ----------
488 ---------- ----------
487 ---------- ----------
498 ---------- ----------
498 ---------- ----------
488 ---------- ----------
476 ---------- ----------
485 ---------- ----------
494 ---------- ----------
480 ---------- ----------
476 ---------- ----------
515 AKVAGF---- ----------
515 AKVAGF---- ----------
509 AKAAGF---- ----------
515 ASDCGFFAAA N---------
475 RASGGTGLDK ----------
523 LSTAMSDRSR NPEPA-----
510 AAAAG----- ----------
526 ---------- ----------
498 ---------- ----------
428 VQPAL----- ----------
427 ---------- ----------
435 ---------- ----------
433 ---------- ----------
506 ---------- ----------
496 ---------- ----------
448 ---------- ----------
437 ---------- ----------
465 ---------- ----------
429 TENSRHTI-- ----------
417 ---------- ----------
415 ---------- ----------
399 ---------- ----------
```

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307    filter
477  EscCol86
477  SalTyp62
477  KlePne26
477  YerPest9
487  VibCho95
486  LisAng27
497  PseAer91
497  PsePut56
487    McoCap
475  983Ferro
484  AciCal19
493  RaoEut12
479  XanCam22
475  TrePal15
520  RhiLeg57
520  RhiEtl15
514  SinMel10
525  RhiSpe81
484  BrdJap46
537  BrdJap47
514  AzrCau10
525  AzsBra35
497  CauCre22
432    MsiTri
426  RhoCap15
434  RhoSph15
432  RhoSph16
505  MyxXant4
495  PlaLimn6
447  LitMon48
436  BaiSu104
464  CloAce37
436  ChaTra59
416  CamJej31
414  HeiPyl44
398  AqiAeo15
```

APPENDIX C:

Nucleotide sequence of extended *mmo* cluster from *Methylosinus trichosporium* OB3b

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APPENDIX C